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The Role of Nitric Oxide Synthase in Mediating Androgenic Gating of Male-Typical Copulatory Behavior in Whiptail Lizards

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The Role of Nitric Oxide Synthase in Mediating Androgenic Gating of Male-Typical Copulatory Behavior in Whiptail Lizards

by

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The Role of Nitric Oxide Synthase in Mediating Androgenic Gating of Male-Typical Copulatory Behavior in Whiptail Lizards

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Male-typical copulatory behaviors such as mounting and intromission are dependent on testicular androgens in most vertebrates, being eliminated by castration and re-instated by administration of exogenous testosterone. Testosterone implants in the preoptic area (POA) can re-instate behavior as effectively as systemic testosterone replacement, implicating this area as a critical locus of hormonal gating. The cellular mechanisms underlying this gating phenomenon are not well understood, but according to one model, testosterone induces an up-regulation of nitric oxide synthase (NOS) in the POA, increasing nitric oxide synthesis following exposure to a sexual stimulus. Nitric oxide in

turn, possibly through its effect on catecholamine turnover, influences the way the stimulus is processed and enables the appropriate copulatory behavioral response. The experiments described in this Dissertation were designed to test this model as it pertains to hormonal gating in *Cnemidophorus* lizards. Specifically, experiments were conducted to test the predictions that nitric oxide synthesis inhibition would suppress the expression of behavior; that preoptic nitric oxide synthesis would be greater in animals expressing copulatory behavior; and that preoptic NOS expression, at both the mRNA and the protein levels, would be greater in animals exposed to testosterone than in animals deprived of hormone. All three of these predictions were upheld, offering support to the model as described.

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Glossary

AR……..Androgen Receptor
AVPV…….Anteroventral Periventricular Nucleus
cGMP…….cyclic Guanosine 3',5'-MonoPhosphate
DHT……Dihydrotestosterone
D-NAME …...inactive isomer of L-NAME
DVR……Dorsal Ventricular Ridge
ER……Estrogen Receptor
ISH…….*In Situ* Hybridization histochemistry
MPN…….. Medial Preoptic Nucleus
MPOA…….Medial Preoptic Area
NADPH……. reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NADPHd……NADPH diaphorase, a marker of NOS-containing cells in the brain
L-NAME…… Nω-nitro-L-arginine-methyl-ester. Inhibits nitric oxide synthase.
NMDAR…….subtype of glutamate receptor with high affinity for N-methyl-D-Aspartic Acid
NO……..……Nitric Oxide
NOS……..…. Nitric Oxide Synthase
nNOS…….... the neuronal isoform of Nitric Oxide Synthase
POA…….. Preoptic Area
PvPOA……. Periventricular Preoptic Area
PR……...Progesterone Receptor
qPCR……. quantitative PCR
RACE…… Rapid Amplification of cDNA Ends
SDN-POA…..Sexually Dimorphic Nucleus of the Preoptic Area
sGC…… soluble Guanylate Cyclase
T…….…..... Testosterone

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Chapter One: Introduction

Among the diverse effects that gonadal sex steroids exert on the behaviors of animals, the power of testicular androgens to activate male copulatory behavior is among the most striking. In vertebrate species with internal fertilization, males in breeding condition will respond to a receptive female by approaching her and initiating a stereotyped series of behaviors leading to sperm transfer. This response is not observed in pre-pubertal animals, whose androgen levels are low, and typically elimination of testicular androgens by castration will completely abolish this display of behavior within a few days or weeks. The behavior can be re-instated by exogenous testosterone administration, which process also requires days or weeks for completion. Furthermore, although testosterone exerts important effects in many parts of the brain, this activation of male-typical copulatory behavior is observed following a localized application of testosterone to the preoptic area (POA). These observations, together with others described below, enable the construction of a conceptual model of behavioral gating by steroids comprising the following components: (i) Sensory information encoding the presence of a receptive female reaches the POA regardless of the hormonal status of the animal. (ii) In the presence of testosterone, some population of cells in the POA changes in such a way as to respond to this information by initiating a

copulatory behavioral response. Clearly, this is not exactly the process that occurs in intact breeding animals, whose brains and behavior presumably change in multifarious ways following systemic testosterone exposure, but it does represent a valuable model with which to test hypotheses about the relationship between hormones, brain and behavior. The experiments described in this dissertation were designed to test one such hypothesis, namely that transcriptional up-regulation of the neuronal nitric oxide synthase (nNOS) gene is one way in which the preoptic area changes following testosterone exposure, so as to increase the probability of a copulatory response to a receptive female. First, brief descriptions are given of the hormones controlling male-typical copulatory behavior and the neuroanatomy mediating its expression, and then possible hypotheses regarding the cellular and molecular substrates of the gating mechanism are discussed.

Hormones controlling male-typical copulatory behavior

Experiments involving castration and hormone replacement have established that testosterone is the primary signal activating male-typical copulatory behavior in a wide range of vertebrate taxa. In rats, the complete disappearance of copulatory behavior takes from several days to several weeks (Davidson, 1966), and its re-instatement requires seven to ten days (McGinnis et al, 1989) of testosterone

exposure. While some of the effects of testosterone are attributable to testosterone binding to the androgen receptor (AR), some part of its action is likely dependent on conversion to estradiol by aromatase in the brain, and subsequent action via the estrogen receptor (ER), although the relative importance of the two pathways varies by species. In rats (Clancy et al, 2000) and in quail (Balthazart et al., 2003), estradiol can activate male-typical copulatory behavior in the absence of testosterone, while the non-aromatizable dihydrotestosterone (DHT) cannot, suggesting that aromatization is critical. In guinea pigs, the reverse is true, DHT being as effective as testosterone in reinstating behavior, and estradiol being ineffective (Alsum and Goy, 1974). In monkeys, while estradiol fails to reinstate behavior in castrated males (Michael et al., 1990), DHT is less effective than testosterone in supporting male-typical copulatory behavior (Michael et al., 1986), and systemic aromatase inhibition does attenuate the effectiveness of systemic testosterone (Zumpe et al., 1993), suggesting that the role of aromzatization is significant. In whiptail lizards, estradiol failed to reinstate male-typical copulatory behavior, and DHT was almost as effective as testosterone (Wade et al, 1993). Aromatization thus does not appear to be a critical component of the hormonal control of male-typical copulatory behavior in this species, and in all the experiments described in this Dissertation, testosterone was used to activate behavior.

Neuroanatomy mediating male-typical copulatory behavior

The neural pathways mediating the expression of male-typical copulatory behavior have been elucidated in some detail in the rat, and several reviews are available on the topic (e.g., Hull et al., 2002). In rodents that rely heavily on olfactory and pheromonal cues to assess the presence and reproductive status of conspecifics, the pathway can be considered to begin at the olfactory epithelia and vomeronasal organs, from which information is transferred via the main and accessory olfactory bulbs to various components of the amygdala. One pathway involving the bed nucleus of the stria terminalis and another via the ventral amygdalofugal pathway bring information from the amygdala to the POA, which also receives projections from various brain regions thought to be associated with other sensory modalities. The motor control of male-typical copulatory behavior can be thought of as essentially a reflex circuit involving sensory feedback from the hindquarters and genitals, which is relayed via the lumbro-sacral spinal cord to motor centers in the brainstem responsible for integrating the muscular movements of copulation. These motor integrating centers, in turn, are under the control of descending pathways from the MPOA and the periaqueductal gray as depicted in Figure 1 (reviewed by Hull et al., 2002).

Testosterone exerts detectable effects on several components of this pathway other than the POA, for example the medial amygdala (Cooke et al, 1999). However, much of the system appears to retain its functional integrity even in the absence of hormone, because testosterone implantation in discrete areas is able to activate male-typical copulatory behavior in an animal deprived of systemic hormone by castration. This suggests that a subset of the system is particularly hormone sensitive, and presumably involved in hormonal gating of behavioral expression. There is some redundancy in the distribution of this function (Wood, 1997), so that testosterone implants in several locations are able to activate behavior, but the POA is particularly sensitive, with preoptic testosterone implants reinstating copulatory behavior in a way that is similar to systemic implants. The conceptual model in Figure 1, according to which testosterone acts at the level of the POA to complete the circuit between the comparatively hormone-independent sensory module upstream and motor module downstream is therefore presented as a reasonable simplification.

The area of the brain labeled “preoptic” is in fact a highly heterogeneous structure in terms of its neurochemistry and its connectivity with other brain areas, and various nuclei and sub-areas within it have been defined (Simerly et al., 1984). Two such areas are particularly relevant to the research described in this Dissertation, one is the area immediately adjacent to the third ventricle, on either

side of the ventricle, at its most rostral extremity. This is the anatomical position of the anteroventral periventricular nucleus (AVPV) in mammals (Simerly and Swanson, 1987), and in whiptails is known as the periventricular preoptic area (PvPOA, Young et al., 1994). Lateral to the PvPOA, and slightly caudal to it is the region known as the medial preoptic area or MPOA.

In mammals the AVPV has been most extensively studied with respect to its role in controlling gonadotropin release (reviewed by Simerly, 1998), and evidence of an important role in controlling copulatory behavior is scarce. In whiptails, the PvPOA strongly expresses AR, ER and PR (Young et al., 1994, 1995) as well as tyrosine hydroxylase (Woolley and Crews, 2004). In rats, nNOS colocalizes with AR and ER throughout the preoptic area, but colocalization is the densest in the AVPV (Sato et al., 2005).

The medial preoptic area (MPOA) has been the focus of research on the neural control of male copulatory behavior for some time (reviewed by Paredes, 2003). It has extensive connections with many areas of the brain, including a massive projection to the AVPV (Simerly and Swanson, 1988).

Cellular and Molecular Mechanisms Mediating Hormonal Gating of Behavior

Steroid hormones can affect cells of the nervous system by several mechanisms, including transcriptional regulation via nuclear receptors, influencing second messengers via membrane-bound receptors, and direct interaction with other signaling cascades (reviewed by Kawata, 1995). Two observations suggest that the first of these mechanisms is involved in androgen gating of male-typical copulatory behavior: in addition to the long time course of behavioral reinstatement by testosterone, preoptic implantation of the protein inhibitor anisomycin eliminates the ability of systemic testosterone to reinstate male-typical copulatory behavior in castrated male rats (McGinnis and Kahn, 1997).

So, if the behavioral changes following testosterone exposure are wrought by changes in gene transcription in cells of the POA, which genes are involved, and how do the cells change? Presumably, for the behavioral response to a given stimulus to change, there must be neurons in the POA whose probability of firing in response to a given input from the sensory module changes following testosterone exposure. Potential mechanisms mediating hormonal gating of behavior can therefore be generated based on mechanisms that are known to

change this probability. Experimental evidence is available for the existence of various kinds of mechanism, of which the majority fall into the following three categories:

1. Mechanisms affecting neural structure

2a. Mechanisms affecting release of neurotransmitter by pre-synaptic cell

2b. Mechanisms affecting the responsiveness of postsynaptic cell to neurotransmitter

Neural Structure

In principle, behavioral gating could be mediated by hormonal induction (or destruction) of synapses, however, disappointingly little empirical data is available to assess the importance of this mechanism. Castagna et al. (1999) report that in the male quail, several ultrastructural aspects of synaptic morphology are sensitive to sex steroids; in particular, the number of axosomatic synapses is reduced by gonadectomy and restored by testosterone. Preoptic expression of GAP-43, a phosphoprotein putatively involved in axonal growth and synapse formation, was increased over control in female rats by four days of estrogen treatment (Singer et al., 1996), suggesting that this protein might be involved in mediating testosterone's effect on neural morphology, although this experiment was not conducted in the context of examining androgenic gating of male-typical copulatory behavior.

Release of neurotransmitters, and responsiveness of cells

For a number of neurotransmitters, a role in influencing male-typical copulatory behavior by effects on the POA has been established by pharmacological means. For a more limited subset, evidence is available to enable assessment of their possible role in hormonal gating. Changes in neurotransmitter release following hormone treatment can be measured directly by microdialysis, and expression of synthetic enzymes can be estimated using immunohistochemical or *in situ* hybridization techniques. The effects of sex steroids on the responsiveness of preoptic neurons to various neurotransmitters can be assessed in a number of ways, including measurement of receptor expression under various hormonal conditions, and comparison of the behavioral or electrophysiological effects of neurotransmitter-specific drugs following hormone administration. In the section that follows, for each of five neurotransmitters, evidence is briefly presented in support of its involvement in mediating male-typical copulatory behavior, followed by what is intended to be a thorough review of available reports of the effect of testosterone treatment on the parameters discussed above.

NOTE: Since the majority of studies of neurotransmitter involvement in male-typical copulatory behavior have been conducted with rats, the behavioral

endpoints reported are necessarily those exhibited by copulating male rats. These include mount latency, intromission latency, intromissions preceding ejaculation, post ejaculatory interval, and so on. Some of these measures have no equivalent in other species (for example those without multiple intromission before ejaculation, such as humans and whiptails), and accordingly hard to interpret in terms of a taxonomically conserved mechanism.

Even more significantly, since the topic of this dissertation is the gating process or neural "decision" to initiate the copulatory sequence, any behavior that follows the first mount can be considered "downstream" of the decision, and therefore not necessarily informative. For this reason, only effects on the mount latency, intromission latency, or proportion of individuals that initiated copulation, or other explicit measure of "sexual motivation" will be considered. Effects on *ex copula* reflexes, intromissive or ejaculatory function will not. Furthermore, since the focus of the discussion is on the POA, the effects of systemic drug administration will not be discussed, and the pharmacological manipulations reviewed in the following section are restricted to intra-preoptic administration, unless otherwise specified.

Glutamate Extracellular preoptic glutamate levels increase in male rats exposed to estrous females, and augmenting this increase by reverse-dialyzing glutamate

uptake inhibitors into the POA facilitated sexual behavior (Dominguez et al., 2006).

Brann et al. (1993) examined the effect of castration and testosterone replacement on NMDA receptor abundance in tissue samples from male rats including the preoptic area and found no effect of either manipulation on either mRNA abundance or NMDA binding.

Norepinephrine Direct infusion of norepinephrine into the MPOA facilitates male-typical copulatory behavior (Mallick et al., 1996) while the non-specific alpha antagonist phenoxybenzamine and the non specific beta antagonist propranolol had the opposite effect. Clark et al. (1985) reported that the alpha2 antagonist yohimbine was able to elicit sexual behavior in castrated male rats, presumably by inhibiting presynaptic autoceptors and increasing norepinephrine release.

The one available report of a study examining androgenic effects on adrenergic receptor expression found no clear effect of testosterone on alpha-2 binding (Balthazart et al., 1991).

Tomaselli et al. (2001) used in vivo microdialysis to measure medial preoptic norepinephrine release in castrated Siberian hamsters implanted with testosterone or cholesterol, and report that norepinephrine release was less in testosterone-treated animals. Gabriel et al. (1988) found that castrating male rats

had no effect on the levels of stored norepinephrine in the POA as measured from tissue dissection by HPLC.

Dopamine Like glutamate, extracellular dopamine levels in the preoptic area of male rats are increased by exposure to a receptive female (Hull et al., 1995). The non-specific dopamine agonist apomorphine restores mounting in castrated male rats when injected into the MPOA (Scaletta and Hull, 1990), while similar injections of cis-flupenthixol, a dopamine antagonist, impair copulation (Pehek et al., 1988). In the quail, there is some evidence that the facilitatory actions of dopamine are mediated by nominally adrenergic receptors (Cornil et al., 2002). Hull et al. (1999), reviewing the effects of preoptic microinjections of receptor subtype specific agonists and antagonists, suggest that different subtypes of dopamine receptors might mediate different phases of the copulatory behavioral sequence. Specifically, they speculate that the intermediate levels of extracellular dopamine observed in the male preoptic area during the initial phase of interaction with a female might act via D_1-like receptors to facilitate parasympathetically mediated penile erection, while the higher levels observed toward the completion of the copulatory interaction might activate D_2-like receptors and favor sympathetically mediated ejaculation.

There are no reports of studies examining the effect of testosterone treatment on preoptic dopamine receptor expression.

Compared to oil controls or DHT treatment, testosterone or estradiol treatment substantially increased the concentration of extracellular dopamine in the preoptic areas of castrated male rats (Putnam et al., 2003). These authors argued that the difference was not due to differential dopamine synthesis, but rather to differential release, possibly mediated by nitric oxide, whose possible role in this process is discussed below.

Serotonin In contrast to dopamine and glutamate, extracellular serotonin levels in the preoptic area of male rats do not vary from baseline during copulatory behavior (Lorrain et al., 1997). Furthermore, a selective serotonin reuptake inhibitor (SSRI) microinjected into the MPOA, did not significantly alter copulatory behavior despite radically increasing serotonin levels (Lorrain et al., 1997), and the 5HT1A agonist 8-OH-DPAT infused into the preoptic area of male rats had no effect on intromission latency (Fernandez-Guasti et al., 1992). On the other hand, the 5-HT1B agonist TFMPP did increase intromission latency when so injected (Fernandez-Guasti et al., 1992), as did serotonin, albeit to a lesser extent. Other workers have found facilitative effects of 8-OH-DPAT (e.g., Matuszewich et al., 1999), but in general they are only robust with respect to the later aspects of copulatory behavior, such as ejaculation. There is some question as to whether the effects of the putative 5HT1A agonist 8-OH-DPAT are indeed mediated by the 5-HT1A receptor (Matuszewich et al., 1999), given that they are

attenuated by co-administration of a D2 antagonist, but not a 5HT1A antagonist (Matuszewich et al., 1999), and furthermore that 5HT1B knockout mice are almost unaffected by 8-OH-DPAT, despite strong effects in wild-type mice (Rodriguez-Manzo et al, 2002).

Preoptic 5HT1A receptor binding is increased by testosterone in the MPOA (Mendelson and McEwen, 1990). Summer and Fink (1998) examined the effect of castration and various hormone treatments on 5HT2a receptor binding and mRNA abundance in the male rat brain. Neither castration or testosterone treatment had any effect on either measure in the preoptic area, despite strong effects in other areas of the brain. . Gabriel et al. (1988) found that castrating male rats had no effect on the levels of stored serotonin in the POA as measured from tissue dissection by HPLC.

GABA The $GABA_A$ antagonist bicuculine when injected into the POA of male rats given low doses of testosterone increases the proportion of individuals exhibiting male-typical copulatory behavior compared with saline controls (Fernandez-Guasti et al., 1986a). Intromission latencies were shortened by $GABA_A$ antagonists, while the proportions of animals mounting were reduced by the $GABA_A$ agonist muscimol and by ethanolamine sulfate (which augments GABA levels by inhibiting GABA transaminase) (Fernandez-Guasti et al., 1986b).

Pennati et al. (2005) report that 3 weeks of exposure to 17alpha-methyltestosterone decreased expression of $GABA_A$ alpha2 and alpha5 subunit mRNA in the preoptic area of male mice.

GABA release in the preoptic area of male rats, as measured by microdialysis was decreased by castration, and this effect was eliminated by testosterone replacement (Tin Tin Win Shwe et al., 2002). Yoo et al. (2000) also report that castration of male rats decreased GABA turnover in the Medial Preoptic Nucleus (MPN) and the Anteroventral Periventricular Nucleus (AVPV), although the effect was somewhat erratic, varying in a non-linear way with time after castration.

Summary of neurotransmitter data Although the available data are insufficient to allow a confident assessment of the likely roles of the various neurotransmitters in hormonal gating, glutamate, acting via increasing dopamine availability, is probably involved. One possible relationship between glutamate and dopamine is discussed in the following section.

Nitric Oxide Nitric oxide is synthesized by Nitric Oxide Synthase enzymes, of which three isoforms have been characterized in vertebrates, the most important isoform in the central nervous system being neuronal nitric oxide synthase (nNOS). The synthetic reaction involves the reduction of a molecule of arginine yielding one molecule of nitric oxide and one molecule of citrulline. While

commonly referred to as a neurotransmitter, differs from the neurotransmitters mentioned above in several significant ways. Rather than being released from vesicles and then binding to a small set of cognate receptors to influence the properties of receptive cells, it diffuses freely though cell membranes, and influences the properties of a wide variety of proteins by at least two mechanisms (reviewed by Ahern et al., 2002). The most well-characterized pathway is via interacting with soluble guanylate cyclase (sGC) to augment the production of cGMP. Cyclic GMP can activate cyclic nucleotide-gated ion channels directly, or, via activation of protein kinase G (PKG) can facilitate the phosphorylation of a number of cell proteins including several ion channels. Nitric oxide can also react directly with cysteine residues of proteins, nitrosylating the thiol group, and such nitrosylation may affect the properties of proteins in diverse ways. For example, S-nitrosylation appears to be responsible for NO-mediated inhibition of the NMDA receptor (Choi et al., 2000).

It was the ability of NO to influence neurotransmitter release, however, that drew researchers' attention to its potential involvement in the neural control of sexual behavior. Having established the influence of preoptic dopamine on male-typical copulatory behavior (Hull et al., 1986), Elaine Hull and her co-workers were stimulated by the report that NO could evoke dopamine release from striatal slices (Hanbauer et al., 1992) to examine the role of NO in

controlling preoptic dopamine release (Lorrain and Hull, 1993). Benelli et al. (1995) reported results further implicating NO in the control of male sexual behavior, including the observation that i.c.v. administration of L-NAME reduced the proportion of individuals mounting. Lorrain et al. (1996) confirmed that the increase usually observed in preoptic extracellular dopamine levels of a male rat following exposure to a female was attenuated by preoptic administration of L-NAME. In this experiment, the NOS inhibitor had no appreciable effect on behavior, but a subsequent experiment (Lagoda et al. 2004) found that suppression of the female-evoked rise in dopamine levels via suppression of NO synthesis by L-NAME was indeed associated with copulatory deficits. Conversely, reverse dialysis of the NOS substrate L-Arginine into the MPOA increases dopamine levels and mounting (Sato et al., 1998). As might be expected, if NO were a significant component of the hormonal gating mechanism, NOS expression is reduced by castration (Du and Hull, 1999), and increased by either testosterone or estradiol (Putnam et al., 2005).

Hull's Model

These and other observations led Hull et al. (2004) to elaborate a model of hormonal gating of male-typical copulatory behavior, according to which testosterone (after aromatization to estradiol) causes up-regulation of nNOS. Glutamate from sensory efferents including those from the medial amygdala signaling the presence of a receptive female stimulates NMDA receptors, through which calcium enters the postsynaptic cells in the preoptic area and activates nNOS via calmodulin. Thus activated, nNOS catalyses the synthesis of NO, which increases the extracellular availability of dopamine, possibly via the cGMP pathway, and possibly by inhibition of the dopamine reuptake transporter. The increased level of dopamine is then postulated to act on unspecified neural substrates to increase the probability of a copulatory response.

Whiptail lizards as experimental subjects for studies of hormonal control of male-typical copulatory behavior

Whiptail lizards of the genus Cnemidophorus offer an unusual challenge to the dogma that male-typical copulatory behavior is dependent on testicular androgens, since some species of the genus are parthenogenic, including only

individuals that are morphologically and endocrinologically female, and yet regularly display male-like "pseudocopulatory behavior" that is indistinguishable from the male-typical behavior of related sexual species (reviewed in Crews, 2005). It is thought that in these animals, progesterone secreted around the time of ovulation may be responsible for activating the behavioral responsiveness to receptive conspecifics that, in males of sexual species, would be activated by testosterone.

This is not such an outlandish hypothesis, given the similarity between the cognate receptors of the two hormones. The steroid receptors in general are a closely related family of nuclear receptor transcription factors (Thornton, 2001), that recognize conserved motifs in the regulatory regions of target genes and affect their transcription (Laudet and Gronemeyer, 2002). In comparison with the other steroid receptors, such as estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR) are particularly similar in structure (Thornton, 2001) and in DNA binding characteristics (Laudet and Gronemeyer, 2002). DNA binding specificity is determined by a structure known as the P-Box, whose amino acid sequence is identical between the AR and the PR (CGSCKV), and this similarity is reflected in overlapping sets of target genes, for example, the AR and the PR both bind the hormone response element of the TAT gene (GGTACAnnnTGTTCT) in a similar manner, while failing to bind the estrogen

response element (canonically AGGTCAnnnTGACCT) (Laudet and Gronemeyer, 2002). It is therefore likely that some of the difference in physiological effects of androgens and estrogens are dependent on the restricted expression of the two receptors to different populations of cells. If so, then progesterone's unusual ability to activate normally testosterone-dependent neural circuits in parthenogenic whiptails might simply be the result of ectopic expression of PR in normally PR-negative, AR-positive cell types in the preoptic area.

No steroid hormone response element has been characterized in the nNOS gene, despite extensive characterization of its regulatory regions (e.g., Jeong et al, 2000; Bachir et al., 2003). A search using Matinspector (Cartharius et al., 2005) of the available flanking regions 5' and 3' to the transcriptional initiation site in the nNOS genes of rat, mouse, and dog reveals a possible ERE at approximately -170bp in the rodent genomes, and a possible ARE at approximately +680bp with respect to the transcriptional initiation site. Neither of these sites, however, is obviously conserved in the other species. A similar search over the twenty kilobases encompassing the nNOS transcriptional start site in the mouse or human genome reveals several possible ERE, ARE and PRE sequences, but without either conservation between taxa or direct experimental verification, it is impossible to attribute functional significance to these sites. In summary, the

bioinformatics results available are insufficient either to rule out direct transcriptional regulation of nNOS by steroids, or to establish its likelihood.

In attempting to understand how progesterone might be able to activate the normally testosterone-dependent pathway in this way, Hull's model of hormonal gating is a particularly attractive one, since it has been known for some time that progesterone control of female copulatory behavior involves nitric oxide (Mani et al., 1994). The idea on which the research described in this Dissertation was based is depicted schematically in Figure 2. Essentially, this model proposes that in the all-female whiptail, periovulatory progesterone induces the up-regulation of nNOS, just as it is presumed to do in the female rat, but does so in an expanded neuroanatomical region, including cells of the preoptic area that are involved in the control of male-typical copulatory behavior. In these cells, the enhanced nNOS activates a neural mechanism usually activated by testicular androgens in males. Since nothing was known about the role of nitric oxide in the whiptail brain, it was necessary to perform some basic experiments regarding the relationship between testosterone, nitric oxide and behavior in these animals, so as to create a foundation for future experiments on progesterone. Accordingly, the Specific Aims of the project were limited to investigating the role of androgen, as follows:

Specific Aims

1. To establish whether nitric oxide is involved in androgen-mediated male-typical copulatory behavior in *Cnemidophorus* lizards, as it is thought to be in rodents. Parthenogenic whiptails expressing male-like pseudocopulatory behavior following androgen exposure were tested with receptive females following injections of a NOS inhibitor or an inactive control.

2. To examine whether nitric oxide is synthesized in the preoptic area during the expression of testosterone-mediated male-typical copulatory behavior. The model illustrated in Figure 2 predicts that NO synthesis in the preoptic area ought to increase during the display of male-typical copulatory behavior. This prediction was tested using citrulline immunoreactivity as a surrogate marker for recent NO synthesis in androgenized and hormone-deprived female lizards.

3. To discover whether nNOS is regulated by testosterone in a manner consistent with a significant role in hormonal gating of male-typical copulatory behavior. If NOS abundance is the proximal factor limiting the expression of male-typical copulatory behavior, then its expression ought to increase with testosterone exposure until a maximum level that coincides with behavioral reinstatement. A group of male lizards was therefore castrated and, after the expression of copulatory behavior had completely subsided, was implanted with testosterone-

filled or empty capsules. At various time points after implantation, subsets of lizards were tested for behavioral reinstatement and then nNOS expression in the preoptic area was assayed by each of three independent techniques.

Chapter Two: The nitric oxide synthase inhibitor L-NAME suppresses androgen-induced male-like pseudocopulatory behavior in whiptail lizards

INTRODUCTION

Male mammals in breeding condition will, in response to a receptive female, display a series of stereotyped behaviors culminating in intromission and ejaculation, while animals deprived of androgens by castration will not. It is thought that the preoptic area (POA) plays a central role in this testosterone-dependent gating (Hull et al., 2002) by a mechanism involving the facilitation of dopaminergic transmission by nitric oxide (NO) (Hull et al., 2004). NO is produced in neurons from arginine by the enzyme nitric oxide synthase (NOS) and is thought to play critical roles in both peripheral and central control of reproductive behavior (Argiolas, 1994). While in male rats, NO is involved in the control of androgen-dependent copulatory behavior (Hull et al., 2004), in female rats, it has been shown to influence progesterone-mediated lordosis behavior (Mani et al., 1994). The involvement of NO in copulatory behavior and its role in mediating the behavioral effects of both androgen and progesterone suggested that it might be involved in the neural control of the unusual male-like copulatory behavior observed in *Cnemidophorus uniparens*, an all-female lizard species. *C.*

uniparens individuals display male-like pseudosexual behavior under the influence of progesterone normally (Grassman and Crews 1986), but can be made to display the same behavior with testosterone treatment (Wade et al., 1993). This display of male-like copulatory behavior is known as pseudocopulation (Crews and Fitzgerald, 1980). The experiment described below was designed to determine whether NO is involved in the male-like pseudocopulatory behavior of whiptail lizards under the influence of circulating androgens.

MATERIALS AND METHODS

Adult *C. uniparens* were captured in the environs of Portal, Arizona, transported to the University of Texas at Austin and housed in environmentally controlled chambers as described by Wade and Crews (1991). Animals were ovariectomized under hypothermic anesthesia and each implanted with a Silastic tube (Helix Medical, 10 mm long, internal diameter 1.47 mm, external diameter 1.96 mm) packed with crystalline dihydrotestosterone (DHT, Sigma). This treatment has been shown to produce male-typical levels of hormone in plasma and results in the robust expression of male-like copulatory behavior (Lindzey and Crews, 1986). Animals to be used as receptive stimulus animals were ovariectomized and after 3 days to recover from surgery were fed three times each week one waxworm injected with 0.5 μg of estradiol (Sigma) dissolved in peanut

oil. This treatment robustly induces receptivity in female and parthenogenic whiptails.

After 3 weeks to recover from surgery, behavioral tests with receptive animals were commenced. The sexual behavior of whiptails lizards has been described by Lindzey and Crews (1986). Tests were conducted by introducing stimulus animals to the subjects' home vivaria (25 cm × 30 cm) with the water bowls and planks removed to leave only the sand substrate. Before conducting tests, each stimulus animal's receptivity was verified by testing with a long-term androgenized stud animal. Behaviors scored for analysis (described by Crews and Fitzgerald, 1980) were APPROACH (subject moves towards stimulus animal and makes contact with her); MOUNT (subject mounts on top of the stimulus animal with forelimbs on either side of her trunk); and PSEUDOCOPULATION (the circular position wrapped around the abdomen of the female that is assumed by males of sexual whiptail species during intromission and ejaculation). Each test lasted 10 min, or until the subject displayed pseudocopulation, and the time at which each behavior occurred was recorded. All animals were given three screening tests and those that failed to mount on at least two of these three tests were eliminated from the study ($n = 7$), leaving ($n = 10$) vigorous courters only. *N*-nitro-l-arginine methyl ester (L-NAME), or its inactive isomer D-NAME (both obtained from Sigma), was dissolved in 0.9% saline and injected intraperitoneally

1 h before the beginning of the test. The target of this experiment was the nitrergic neurons innervating the preoptic area, but L-NAME exhibits little specificity for any one isoform of nitric oxide synthase, so in addition to its effects on brain mechanisms other than those mediating sexual behavior, it affects a number of other physiological processes such as the control of blood pressure. Pilot experiments were therefore conducted to establish a dose that affected sexual behavior without being broadly debilitating. L-NAME administered at 600 μg per individual was effective in suppressing copulatory behavior, while doses greater than this appeared to have effects on the animals' behavior other than on copulatory endpoints, as assessed by observing each animal's ability to seize a live, moving cricket presented after the termination of the test. Generally, unmanipulated lizards will seize this prey avidly, and successful capture within a few seconds was taken as evidence of intact motor and motivational systems.

All animals were first given a baseline test without drug treatment to establish typical latencies to the three behavioral endpoints. Each subject was then tested on two more occasions, once 5 days later and once 2 days after that (i.e., 5 and 7 days after the first test). Each subject was tested after treatment with L-NAME on one of these later tests, and after treatment with D-NAME on the other test. Order of drug versus control treatment was balanced across subjects.

RESULTS

All experimental animals regardless of treatment seized crickets following their tests within 5 s (data not shown). Figure 3 shows mean approach, mount, and pseudocopulatory posture latencies for the three testing conditions, and shows that animals responded in a similar manner when treated with D-NAME as they did in the baseline test. For this figure, animals that did not show a behavior were assigned the maximum latency of 600 s. All animals approached, mounted, and achieved the copulatory posture when treated with D-NAME, as was the case in the baseline test. When treated with L-NAME, half the animals failed to mount. All animals that mounted despite L-NAME treatment also went on to achieve the pseudocopulatory posture. The expression of this latter behavior being dependent on mounting, all animals that failed to mount also failed to show pseudocopulation. The effect of L-NAME treatment on approach behavior was somewhat weaker; in L-NAME trials, 8 out of 10 subjects approached the stimulus animal, compared to 10 out of 10 during the D-NAME and baseline trials. Latencies for the three behaviors appeared not to differ between the three different trials; although half the animals failed to pseudocopulate when given L-NAME, the individuals that did express the behavior did so with similar latencies whether given L-NAME, D-NAME, or untreated. For each behavior, latencies were subjected to repeated measures ANOVA for all individuals that did express the

behavior in all three trials (individuals that did not express a behavior were not included in this analysis because the assignment of the full 600 s latency for animals not expressing a behavior was considered too arbitrary). For APPROACH, $n = 8$, $F = 0.18$, $P > 0.841$; MOUNT, $n = 5$, $F = 0.33$, $P > 0.73$; for PSEUDOCOPULATION, $n = 5$, $F = 0.61$, $P > 0.565$. Since the principal difference between L-NAME and other groups was the proportion of individuals that exhibited the various behaviors, the categorical responses under the three conditions (pre-test, D-NAME, or L-NAME) were subjected to Cochran's Q test for each behavior; APPROACH was not significantly different between the treatments ($P < 0.135$); mounting and pseudocopulation were both observed less frequently in animals that had been treated with L-NAME ($n = 10$, $P < 0.007$).

DISCUSSION

The suppression of pseudocopulation by L-NAME in this species is consistent with the idea that NO synthesis may be involved in the control of expression of this behavior. Results of reported experiments examining the effects of NO synthesis inhibition on male copulatory behavior in rats are somewhat heterogeneous (see Hull et al., 2002). One source of variation in reported results is probably the effect of previous sexual experience. Benelli et al. (1995) found that systemic L-NAME treatment reduced the percentage of individuals mounting and

ejaculating in both experienced and naive rats, while intracerebroventricular administration had this effect only in naive animals. Bialy et al. (1996) report that the impact of L-NAME treatment was largely on the later consummatory aspects of the mating sequence, suggesting that sexual motivation was intact, while Ratnasooriya et al. (2000) found that L-NAME caused a marked reduction in precopulatory behaviors such as pursuit and anogenital investigation, suggesting the opposite. The results of a recent thorough investigation of the influence of experience on the effect of central NOS inhibitor administration in male rats (Lagoda et al., 2004) suggest that while sexual experience does attenuate the effect of NOS inhibition on the display of mounting, even sexually experienced animals show greatly decreased levels of consummatory copulatory behaviors after infusion of L-NAME to the medial preoptic area. The animals in the present study had been given the opportunity to express pseudosexual behavior on at least the three screening tests, in addition to an unknown amount of pseudosexual experience in the wild before their capture, and are perhaps best considered "experienced" for the purposes of this discussion. This interpretation is consistent with the observations of Lagoda et al. (2004), who found that while in naïve male rats mounting was almost eliminated by L-NAME treatment, in experienced animals, slightly less than half exhibited mounting despite L-NAME treatment, a proportion similar to that seen in the present study. What, mechanistically, is

different between experienced and naïve rats, or between those experienced animals sensitive to NOS inhibition and those that appear refractory to such treatment, remains an interesting question.

Another important confound that is latent in experiments involving systemic injection of NOS inhibitors is the effect of the drug on peripheral physiology, particularly penile erection. Since NO synthesis is critically involved in the local control of erection (Cartledge et al., 2004), the effect of its systemic suppression on male copulatory behavior is only partly attributable to mechanisms in the central nervous system. Two observations make it likely that the suppression of male-like copulatory behavior in *C. uniparens* is independent of penile erection. Firstly, the animals that failed to pseudocopulate did so because they failed to mount, suggesting that the deficit was more in motivation than in consummatory ability. Secondly, these animals have entirely female-typical morphology and no erectile or intromittent structures with which the drug might interfere. The results observed are therefore compatible with an effect of NO synthesis suppression on the central mechanisms of appetitive motivation. A significant problem in thus attributing the observed effect, particularly given the fact that suppression of NO synthesis was not verified other than by the observation of the behavioral effect, is that the drug L-NAME, which is not specific to the neuronal isoform of NOS, also has the potential to interfere with

other physiological systems that depend on NO synthesis. When administered systemically, the contribution of such unintended effects to a behavioral observation cannot be ruled out, although the fact that the animals' normal feeding behavior was unimpaired argues against such an interpretation in this case.

Chapter Three: Testosterone induction of male-typical sexual behavior is associated with increased preoptic NADPH diaphorase and citrulline production in female whiptail lizards

INTRODUCTION

In vertebrates with internal fertilization, males in breeding condition will respond to receptive females with a sequence of stereotyped courtship and copulatory behaviors culminating in sperm transfer. These male-typical behaviors are dependent on gonadal sex steroids, particularly testosterone, so that the levels of these behaviors displayed by animals following castration will diminish over a period of days and, in most individuals, disappear completely. In castrated, previously sexually active males, mating behavior can be reinstated by the administration of exogenous testosterone. This re-establishment also requires several days to complete, although the latency is dependent on the time elapsed since castration, sexual experience, and several other factors. The neuroanatomical substrates of male copulatory behavior have been extensively mapped in rodents, focusing on the pathways involved in the transmission of pheromonal and other olfactory information from the main and accessory olfactory bulbs via the olfactory component of the amygdala to the preoptic area, and thence to areas of the brainstem putatively controlling copulatory motor patterns. Because testosterone implants in the preoptic area can re-establish

copulatory behavior in an animal systemically deprived of hormone, this area has been a focus of attention for researchers attempting to elucidate the cellular changes underlying the hormone-mediated gating of copulatory behavior. According to one current model, the gating mechanism involves a testosterone-dependent increase in dopamine availability in the preoptic area. Hull and coworkers (e.g., Dominguez and Hull, 2005) have proposed that dopaminergic stimulation of preoptic neurons alters the processing of afferent information about the presence of a sexual stimulus. In turn, efferent signals from the preoptic area to the brainstem motor areas controlling copulatory behavior are altered in such a way as to augment the probability of a copulatory behavioral response. The same model posits that dopamine availability is influenced by nitric oxide (NO), and that an increase in the expression and action of the neuronal isoform of NO synthase (nNOS), the enzyme responsible for NO synthesis, is an important effect of testosterone's action in the preoptic area. Castration decreases NOS expression in the medial preoptic area in rats (Du and Hull, 1999) and Syrian hamsters (Hadeishi and Wood, 1996). Furthermore, pharmacological inhibition of this enzyme suppresses copulatory behavior in previously sexually active male rats (Benelli et al., 1995; Ratnasooriya et al., 2000), lending support to the idea that NO synthesis is an important component of the androgen dependent gating of male sexual behavior.

Whiptail lizards of the genus Cnemidophorus offer some revealing insights into the androgen control of male-typical copulatory behavior (discussed by Crews, 2005). The genus includes normal gonochoristic species such as C. inornatus, and also several parthenogenic species. One of the parthenogenic species, C. uniparens, has been studied extensively because despite having all of the morphological and physiological characteristics of females, at certain times in the ovarian cycle, these animals exhibit towards receptive conspecifics a suite of copulatory behaviors identical to those shown by male whiptails. Male-like behavior can also be elicited from these animals in the laboratory by androgen treatment, but no androgens are detectable in unmanipulated parthenogens or females. Rather, the display of male-like behavior in the parthenogen is thought to be dependent on progesterone, which peaks around the time of ovulation when male-like behavior is observed. This unusual hormone-behavior relationship has stimulated the search for components of the neural system controlling male-typical copulatory behavior that might be targets of either androgens (in males or androgen-treated females) or progesterone (in parthenogens or progesterone-treated males). The NO system is a good candidate for such a target because it is androgen-regulated, involved in controlling male-typical copulatory behavior (as discussed above), and is known to be influenced by progesterone (Mani et al., 1994). A previous experiment had established that pharmacological inhibition of

NO synthesis suppresses testosterone-induced male-like copulatory behavior in C. uniparens (Sanderson et al., 2005). The experiment described here was designed to test two predictions concerning the effect of testosterone treatment that induces male-like behavior in female whiptails: first that it would up-regulate NOS expression in the preoptic area and second that it would increase NOS activity in the same area following male-like copulatory behavior.

Two endpoints were compared between testosterone- treated females and controls: numbers of putatively NOS-containing cells were examined using NADPH diaphorase (NADPH-d) histochemistry, and NOS activity was assessed using citrulline immunohistochemistry. In the brain, NADPH-d is considered to be a reliable marker of nNOS, producing comparable distributions of stained cells to those marked by nNOS immunohistochemistry (Dawson et al., 1991), and has been used to mark nitrergic neurons in diverse vertebrates including the tilapia (Bordieri et al., 2003), the pigeon (Cuthbertson et al., 1999), and the gecko (Smeets et al., 1997), as well as rodents and primates. Citrulline is produced from arginine concomitantly with the synthesis of NO under the catalytic influence of NOS, and because other sources of the amino acid in the brain are not abundant, citrulline immunoreactivity is a reliable marker of NO production in the period immediately preceding sacrifice (Keilhoff et al., 2000; Martinelli et al., 2002). In order to distinguish a possible testosterone-induced increase in the activity of

nitrergic cells mediating copulatory behavior from changes in activity in cells mediating other processes, citrulline immunoreactivity was examined in subsets of animals that had been exposed to sexual or nonsexual stimuli immediately before sacrifice.

MATERIALS AND METHODS

Animals and Hormone Treatments

Female C. inornatus were captured in the vicinity of Sanderson, Texas. They were held in environmentally controlled chambers over the winter of 2004 as described by Wade and Crews (1991) and in May of 2005 were ovariectomized and implanted with 12 mm Silastic implants containing crystalline testosterone or an identical capsule containing no hormone. Such testosterone implants have previously been shown to produce systemic testosterone levels typical of breeding males in female whiptails (Lindzey and Crews, 1986) and to elicit male-typical copulatory behavior (Wade et al., 1993).

Behavioral Testing

Five weeks after testosterone implantation, each animal was given a single test, either with a female whiptail made receptive by intramuscular injection of 0.5 g of estradiol dissolved in peanut oil, or else with a dummy comprising a plastic tube approximately the same size as a lizard. All animals with blank

implants were given a test with a receptive female (BLANK+F group, n = 8), while animals with testosterone implants were divided into two groups, a T+DUMMY group (n = 8) tested with the dummy and a T+F group (n = 9) tested with receptive females. Animals' water bowls and planks were removed 10 min before the test to facilitate observation, and the test itself was allowed to proceed for 10 min after the introduction of the female. Latencies to the exhibition of mount and intromissive posture as described by Crews and Fitzgerald (1980) were recorded for those animals displaying these behaviors.

Perfusion and Tissue Processing

Immediately after the end of the 10 min testing period, the experimental animal was removed and injected with an overdose of sodium pentobarbital before perfusion through the ventricle with 10 mL of 0.9% saline and 10 mL ice-cold fixative compared of 1% glutaraldehyde, 0.2% sodium metabisulfite, and 3% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. Brains were dissected from the skulls, post-fixed in the same fixative at 48C overnight, and then transferred to 30% sucrose in PBS for 2 to 3 days. After the brains sank they were blotted to remove excess sucrose, frozen in isopentane cooled with dry ice, and stored at 808C. Brains were then mounted on a cryostat chuck in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) and cut at 40 m on an HM500 cryostat (MICROM, Waldorf, Germany). Sections were cut in four

series into antifreeze containing 30% sucrose, 30% ethylene glycol, and 1% polyvinyl pyrrolidone in PBS and stored at 208C. During sectioning, one brain was destroyed by a cryostat malfunction.

NADPH-d Staining

One series of sections from each brain was retrieved from antifreeze and rinsed in Tris-buffered saline (TBS), pH 7.4, before incubation at 378C for 100 min in TBS containing approximately 0.5 mM NADPH (N-7505; Sigma), 0.4 mM Nitro Blue Tetrazolium (N-5514; Sigma), and 0.3% Triton- X. Sections were then washed in TBS at room temperature and stored in the same buffer at 48C until mounting. Control sections were subjected to the same treatment except that the NADPH was omitted.

Citrulline Immunohistochemistry

Sections for citrulline immunohistochemistry were rinsed in PBS, incubated for 25 min in 75% methanol, 0.075% hydrogen peroxide, then in 0.5% sodium borohydride, 0.2% sodium metabisulfite for 1 h before blocking in PBS containing 0.3% Triton-X (PBST), 1% nonfat milk solids, and 10% normal goat serum. After 5 h blocking, during which period the primary antibody (rabbit anti-citrulline, AB6464; Abcam U.K.) was pre-incubated with the same blocking solution, sections were transferred to primary antibody at a final concentration of 1:5000 and incubated for 12 h. Sections were then extensively washed in PBST,

before incubation for 1 h in biotinylated goat anti-rabbit antibody (BA-1000; Vector Laboratories, Burlingame, CA) diluted 1:1000 in PBST containing 5% normal goat serum, and then 1 h in horseradish peroxidase-conjugated Avidin Biotin Complex (PK-6100; Vector) diluted to one-fifth of the manufacturer's recommended concentration with PBS, 0.1% Triton-X, and 0.05% bovine serum albumin. Staining was developed with the DAB substrate kit (SK-4100; Vector) following the manufacturer's directions. All steps were carried out at room temperature. Omission of primary antibody eliminated staining entirely. Of the first series to be processed, some slides were counterstained with toluidine blue to assist in locating regions of interest, and because the counterstaining interfered with optical density measurement, a second series was immunostained in the same way as the first, and all measurements were taken from this second series.

Cell Counting and Optical Density Analysis

Cells stained by NADPH-d histochemistry were counted using the optical fractionator routine included in the Stereo Investigator program (Microbrightfield, Williston, VT). The preoptic area, including the periventricular and medial preoptic areas, was delineated on the computer screen according to the atlas of Young et al. (1994) using the 10X objective of a Zeiss microscope, and cells included in counting frames spaced systematically by the computer within this area after a randomly selected start point were marked using the 100X oil

immersion objective. Two sections were counted for each brain, a smaller number than is common for stereological studies (Mouton, 2002), because of the small size of the lizard preoptic area. For citrulline immunostaining, which was expected to be distributed through the neuropil rather than confined to cell bodies, optical density was measured from micrographs taken with standardized illumination using a 10X objective on an Olympus microscope. Digital micrographs captured with a Microfire camera (Microbrightfield) and Picture-Frame software, also supplied by Microbrightfield, were converted to eight-bit grayscale JPEG files and analyzed using ImageJ software (Abramoff et al., 2004) on a Macintosh G4. ImageJ was used to delineate an area of interest corresponding to the preoptic area, to eliminate areas of the image corresponding to holes in the tissue or artifacts due to dirt, and to measure the optical density in comparison with a Kodak step tablet (Rasband, 1997–2005). Two sections through the preoptic area were sampled for each brain: one section just caudal to the rostral extremity of the third ventricle, and the section immediately caudal to that, which was generally the section in which the anterior commissure was first observed. Slides were coded for quantitative analyses and the microscopist was blind to the experimental groups. After decoding the slides one coded identity was found not to correspond to an experimental subject and was not included in any

further analysis. All statistical analysis was performed with the SAS program (SAS Institute, Cary, NC) on the University of Texas Windows Server.

RESULTS

Behavior

All testosterone-implanted individuals tested with a receptive female mounted the stimulus animal with latencies varying from 22 to 442 s (median = 114), and proceeded to assume the intromissive posture with latencies of 135 to 540 s (median = 245). No animal in either BLANK or DUMMY group mounted the stimulus animal.

NADPH-d

This technique produced vivid blue staining of cells and fibers in several areas of the brain, while control sections lacked the blue formazan staining characteristic of the technique. Distribution of stained cells and fibers was essentially similar to that reported by Smeets et al. (1997) for the gecko, with well-stained cells obvious in the striatum, the dorsal ventricular ridge, the amygdaloid complex, and the diagonal band of Broca, although stained cells were somewhat less numerous and fiber plexuses less dense than reported by Smeets et al. (1997). The target population of cells in the preoptic area was generally more lightly stained than other obvious populations of nitrergic cells, and was mostly

arranged close to the third ventricle Figure 4B. Total numbers of these periventricular NADPH-d-positive cells were estimated based on the density in the sampled sections and the estimated volume of the region of interest, and compared using the Wilcoxon test because of heterogeneity of variance and deviation from normality of the data. No difference was expected in this parameter between the T+DUMMY group and the T+F group, because they only differed in the nature of the test they were given immediately before sacrifice, and none was observed ($p = 0.740$). Accordingly, data from these two groups were pooled and compared with the BLANK+F group. Estimated cell numbers were significantly higher in the testosterone-treated groups than in the blank group ($p = 0.0002$; Fig. 4).

Citrulline Immunostaining

In several areas of the brain, particularly the more rostral ventral forebrain, very clear staining of cells and fibers was observed with this technique. In the preoptic area that was the focus of this study, dark, diffuse staining was observed, with a few well defined cells close to the ventricle in a subset of the brains (Fig. 5B). The degree of staining was graded medio-laterally and rostro-caudally, with the darkest staining being apparent close to the ventricle and towards the rostral extremity of the preoptic area. Because optical density was almost universally greater in the more rostral of the two sections, optical densities were analyzed

with a mixed model with section position (rostral or caudal) as a within-subject variable, using the MIXED procedure of the SAS program. Citrulline immunoreactivity as measured by optical density was substantially greater in the rostral preoptic area than in the caudal ($p = 0.0001$), but the interaction between rostro-caudal position and group was not significant. Accordingly, the main effect of group was examined with planned comparisons of the BLANK+F group with each of the testosterone-treated groups (Fig. 5C). This analysis suggested that citrulline immunoreactivity was significantly higher in the T+F group than in the BLANK+F group ($p = 0.011$), but that the difference between the BLANK+F group and the T+DUMMY group was not statistically significant ($p = 0.289$).

DISCUSSION

If the NADPH-d staining is considered a reliable marker of nNOS expression, the present study demonstrates a robust effect of testosterone treatment up-regulating preoptic nNOS, consistent with results from the rat and hamster (Du and Hull, 1999; Hadeishi and Wood, 1996). This was not unexpected, because the NOS inhibitor L-NAME suppresses the expression of male-like copulatory behavior in androgen- treated C. uniparens (Sanderson et al., 2005), implicating the enzyme in the hormonal control of this behavior in this species. However, it has recently been reported that testosterone treatment has no effect on

NOS expression in the preoptic area of male quail (Martini et al., 2005), and indeed that very little NOS immunoreactivity is observed in the medial preoptic area in this species. These observations prompted Martini et al. (2005) to speculate that the role of a NOS-dependent mechanism in controlling male-typical copulatory behavior might be limited to some subset of vertebrate taxa including rodents but excluding birds. The results of the present study demonstrate that whiptail lizards exhibit the pattern of testosterone up-regulation of preoptic NOS observed in rodents, and suggest that the set of taxa in which NOS has this role is a large one.

The pattern of putatively nitrergic, NADPH-d-positive cells and fibers revealed in this experiment was similar to that reported by Smeets et al. (1997) for the gecko. For the purposes of the present study, the most important difference was that the technique stained noticeably fewer cells in the preoptic area in this study than were observed by Smeets et al. (1997). These authors reported observing several rows of NADPH-d-stained cells running parallel to the third ventricle, which is indeed what is observed with this technique in our hands using tissue that has been less extensively fixed. In this study NADPH-d-stained cells were distributed at the same distance from the ventricle as were the rows, but were less numerous, and generally the row structure was not very clear. As the intensity of fixation increases, the number of cells marked decreases (unpublished

observations), and the simplest interpretation of the quantitative discrepancy between the results of Smeets et al. (1997) and our own is that NADPH-d activity was reduced by the intensive fixation in paraformaldehyde and glutaraldehyde used in the present study because of the requirements of the citrulline immunohistochemical technique (the citrulline antibody is raised against a citrulline-glutaraldehyde-protein conjugate and requires glutaraldehyde fixation of tissue to ensure antigenicity). It is thus possible that the study systematically underestimated the total number of NOS-containing cells.

The fact that citrulline immunoreactivity was greater in testosterone-implanted females than in blank-implanted females, but only following exposure to a sexual stimulus, strongly supports the hypothesis that an up-regulation of NO production is involved in the androgen-induced expression of male-typical copulatory behavior. The fact that citrulline immunoreactivity was intermediate in animals implanted with testosterone but not exposed to a sexual stimulus leaves open the possibility that NO production in this region of the brain following testosterone exposure may be up-regulated constitutively, in addition to the activity elicited by sexual behavior. A firm conclusion on this matter will require a study of sufficient statistical power to reveal definitely whether the testosterone-treated, sexually unexposed group resembles one of the other groups, or is genuinely distinct from either. In female rats, mounting behavior and aggression

are both activated by testosterone (van de Poll et al., 1986). The question therefore arises whether the mounting behavior displayed by testosterone-treated females exposed in this experiment to receptive females might be agonistic in nature, rather than copulatory. By extension, the putative NO production inferred from the increased citrulline immunoreactivity in the preoptic areas of the mounting animals might also be interpreted as aggression-related. This possibility was not considered in the design of this experiment, because the behavioral endpoints included the characteristic intromissive posture as well mounting. This behavior is not observed in agonistic contexts in this species (Crews et al., 1983; Lindzey and Crews, 1988), and because it was displayed toward the receptive stimulus female by every individual in the T+F group, it seemed reasonable to interpret the response as being copulatory in nature. However, agonistic behaviors share with male-typical copulatory behavior several key characteristics, including activation by androgens (van de Poll et al., 1986) and the involvement of NO and serotonin (Chiavegatto and Nelson, 2003), and discrimination between the two would require explicit comparison of the supposedly copulating group with a group exhibiting unambiguously aggressive behavior.

The distinction between NOS activity following copulatory behavior and constitutive activity is an important one. It is thought that most nNOS activity is dependent on calcium influx resulting from the activation of an NMDA receptor

physically coupled to the enzyme by scaffolding proteins (reviewed by Kiss and Vizi, 2001). This suggests that specific glutamatergic afferents to an area might activate one set of NMDA receptors, and therefore one set of NOS moieties, while another set of afferents might affect a different population. Presumably, therefore, the cells in which NOS might be up-regulated by testosterone exposure, and subsequently stimulated with glutamatergic input during exposure to a sexual stimulus, are not the same set of cells that would produce NO if the same area of the brain were homogenized and assayed in vitro. In fact, there is good evidence that NOS in the preoptic area is activated during male-typical copulatory behavior by glutamatergic afferents from the medial amygdala (Dominguez et al., 2001). Although, to our knowledge, no previous studies have assessed the effect of castration or testosterone administration on NOS activity in the preoptic area following a copulatory stimulus, two have assayed ex vivo NOS activity in samples including preoptic tissue (Singh et al., 2000; Reynoso et al., 2002). In both cases, the authors reported that castration in fact decreased NOS activity or had no effect, but neither study was designed to examine the role of NO synthesis associated with sexual behavior. On the other hand, using microdialysis in awake, moving rats, Pu et al. (1996) found that NMDA infusion increased cGMP release (a marker of NO production) in intact but not in castrated males. This result agrees well with the results of this experiment, in which glutamatergic stimulation

can be thought of as having been provided endogenously in response to the sexual stimulus. In summary, results of the present study confirm and extend evidence from other species suggesting that an increase in NO synthesis capacity is a significant component of the androgen-dependent gating of male-typical copulatory behavior.

Chapter Four: Neuronal nitric oxide synthase induction in the preoptic area by testosterone is consistent with a role in gating male copulatory behavior

INTRODUCTION

Expression of male-typical copulatory behaviors such as mounting and intromission is activated by testicular androgens in most vertebrates, being eliminated by castration, and reinstated by exogenous testosterone (Hull et al., 2002). Some component of the preoptic area (POA) appears to be critical in the hormonal gating of the behavior, since testosterone implants in this locus will reinstate behavior in castrated animals as effectively as systemic implants (Wood, 1998). The mechanism may involve local aromatization of testosterone, and some effects of testosterone can be mimicked by estradiol (Hull et al., 2002).

According to one influential model (Hull and Dominguez, 2006), the probability of a copulatory response to a receptive female is influenced by dopamine availability in the medial preoptic area (MPOA). Extracellular dopamine levels are increased by nitric oxide (NO), whose levels are determined by the enzyme neuronal nitric oxide synthase (nNOS). This enzyme is associated with the intracellular domain of the NMDA receptor, and its activity is dependent

on calcium, via calmodulin. Calcium influx sufficient to activate the enzyme occurs following the activation of NMDA receptors by glutamate from amygdalar efferents conveying information about sexual stimuli. Once activated, the enzyme produces NO, which diffuses through the cell membrane and inhibits the dopamine reuptake transporter on neighboring varicosities, increasing extracellular dopamine levels and enabling behavioral expression. The gating effect of testosterone is thus achieved via regulation of the abundance of nNOS.

A basic prediction of this model is that preoptic nNOS ought to diminish following castration and be up-regulated following androgen replacement. Du and Hull (1999) found that castration of male rats decreased the number of nNOS-expressing cells in the medial preoptic nucleus (MPN). Putnam et al. (2005) reported that either testosterone or estradiol increased the number of nNOS-immunoreactive cells in the MPOA relative to vehicle or dihydrotestosterone in castrated male rats. Scordalakes et al. (2002), found that exposure to testosterone or estradiol resulted in greater nNOS immunoreactivity in gonadectomized wild type mice than in estrogen receptor knockouts, suggesting that testosterone up-regulates nNOS via the estrogen receptor, after aromatization. However, the only study to our knowledge to examine the effect of androgen on preoptic *nNOS* mRNA found that castrating rats increased expression in preoptic tissue samples, while androgen decreased it (Singh et al., 2000).

The present experiment was designed to resolve the apparent discrepancy between androgens' effects on preoptic nNOS protein and mRNA. Because the response of the gene to testosterone might differ according to anatomical location, the study was restricted to a periventricular population of nitrergic cells that has been previously implicated in androgen gating of copulatory behavior in the whiptail lizard (Sanderson et al., 2006). Transcriptional induction was assessed by quantitative PCR following laser microdissection to enable reliable quantitative measurement and precise anatomical targeting. Adjacent sections were labeled by *in situ* hybridization to corroborate the qPCR data, or by NADPH diaphorase histochemistry to assess protein expression. To enable comparison between gene expression and behavioral gating, measurements were made in the context of a castration and hormone-replacement paradigm.

MATERIALS AND METHODS

Animals, Hormone Treatment and Experimental Design

Adult *Cnemidophorus inornatus* were captured in the vicinity of Sanderson, TX and transported to the University of Texas Austin campus, where they were individually housed in environmentally controlled chambers in terraria with *ad libitum* water and food in the form of crickets. After one to two weeks to

acclimatize to the laboratory environment, they were castrated as described by Wade and Crews (1991) and returned to their original tanks. Seven weeks later they were given three ten-minute tests with receptive females to verify the absence of courtship behavior, and then implanted subcutaneously with 12mm Silastic implants packed with crystalline testosterone, or with empty implants. Such testosterone implants have been shown to produce physiologically relevant serum hormone levels and to induce male-typical copulatory behavior (Lindzey and Crews, 1986). Controls were implanted with empty capsules. One, three, nine, or eighteen days later, animals were tested with receptive females for reinstatement of copulatory behavior and then immediately sacrificed. Sexual experience was thus equivalent among all groups, other than the test immediately before sacrifice, which involved copulation for some animals and not others. After sacrifice, brains were sectioned as described below in four series to enable independent assays of *nNOS* mRNA and protein expression in adjacent sections from the same animals. All procedures were approved by the University of Texas IACUC.

Sectioning and Microdissection

Brains were rapidly dissected from skulls, frozen in tissue freezing medium on dry ice, and then stored at -80°C until cryosectioning. Brains were sectioned at 20 microns in four series, of which three were thaw-mounted onto

superfrost plus slides (Fisher) otherwise held at -20°C in the cryostat, and one was similarly mounted onto membrane-covered slides for laser microdissection (P.A.L.M. Microlaser Technologies AG, Germany). Before use, these membrane-coated slides were prepared according to the supplier's instructions, including two hours' baking at 180°C to inactivate RNAses, 30 minutes' UV exposure under a PCR hood to decrease hydrophobicity, poly-L-lysine treatment and air drying. Laser microdissection was performed immediately after sectioning. Slides for *in situ* hybridization or NADPHd were stored at -80°C for several weeks before use.

Behavioral Observations

Animals were tested in their home tanks with stimulus females rendered receptive by injection of 0.5 µg of estradiol on two sequential nights before the test. Tests followed a standard paradigm of ten minutes of interaction with the female during which latencies to approach and mount behaviors were recorded if they occurred (Crews and Fitzgerald, 1980).

Cloning of Whiptail *nNOS*

A 900bp fragment of *nNOS* was cloned from whole-brain lizard cDNA by a degenerate PCR strategy using primers based on available human, teleost, and Xenopus sequences. A radioactively labeled sub-clone of this fragment was used to probe a lizard brain cDNA library (Lambda ZAP II, Stratagene, La Jolla, CA), from which a 2155 bp fragment was retrieved. An additional 892 bases

corresponding to the 5' end of the coding region and 5' untranslated region was cloned by 5' RACE (Clontech, Palo Alto, CA, cat # 634914) according to the manufacturer's instructions. Sequence is available under Genbank accession number DQ141603.

Laser Capture Microdissection and RNA isolation

Sections on membrane-covered slides were taken directly from the cryostat, fixed for five minutes in 70% ethanol at -20°C, dipped in 20% ethanol, stained with 0.5% toluidine blue for two minutes, rapidly dehydrated in ascending ethanols and air-dried in a laminar flow hood. Sections were then visualized on a microdissection microscope (P.A.L.M. Microlaser Technologies AG, Germany), and an area corresponding to the rostral periventricular preoptic area excised and captured with the laser (see Figures 6 and 8). This target area was generally found on two or three sequential sections in each brain. Once capture was complete, 30 µl of TRIZOL (Invitrogen, Carlsbad, CA) was added to the caps in which the fragments were captured, incubated for five minutes, and then spun down into the tube, after which total RNA was isolated according to the manufacturer's instructions.

Reverse Transcription and Quantitative PCR

Each RNA sample was treated with DNAse I (Ambion, Austin, TX) according to the manufacturer's instructions, with the addition of 20 units of

RNAse inhibitor (RNAse OUT, Invitrogen) to degrade genomic DNA contamination. The resulting RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) in a 40 µl reaction using a mixture of oligo dT primers and primers specific for *nNOS* and 18S RNA. These gene-specific primers were designed to be 100-200bp 5' to the primer used in quantitative PCR on the non-coding strand. Positive controls used 1ng of whole brain RNA in place of RNA derived from laser microdissected samples, and in negative controls the reverse transcriptase was omitted. Samples were incubated at 50°C for 60 minutes, the reaction was terminated by incubation at 70°C for 12 minutes, and excess primers and salts were removed by dilution and re-concentration in Microcon YM30 columns (Millipore, Bedford, MA).

For each laser microdissected sample, 18S RNA abundance and *nNOS* abundance were each measured in triplicate in an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.2.1 software), using SYBR Green I dye (Invitrogen). Primers for 18S were designed from the *Cnemidophorus* 18S rRNA (Genbank AY217941, bases 1173-1287), and for *nNOS* from the cloned sequence described above (bases 1638-2278). Standard curves were constructed using known dilutions of cDNA, and amplification efficiency calculated for the two primer pairs. For each individual, median values from the 18S and *nNOS* triplicates were used to calculate the relative transcript abundance of *nNOS* using the Mean

Normalized Expression formula of Simon (2003). For two individuals, the reference gene signal was less than the smallest value of the standard curve, and these individuals were excluded from further analysis. After amplification, qPCR products were sequenced on both strands to confirm the identity of the target.

Northern Hybridization

A 641bp fragment of the *nNOS* sequence described above (bases 1638-2278) was subcloned into pCR II-TOPO, and a digoxygenin-labeled probe prepared by *in vitro* transcription using Ambion's Megascript kit. To check its specificity, five micrograms of total RNA extracted from whole lizard brain was electrophoresed through a formaldehyde agarose gel, and then electroblotted onto positively charged nylon membrane. After cross-linking with U.V. light, the membrane was prehybridized with Ultrahyb (Ambion, Austin, TX) and then probed with approximately 10 ng/ml of *nNOS* probe. After hybridizing at 68°C overnight and washing, the probe was visualized with the CDPstar chemiluminescent system (Roche Applied Science, Indianapolis, IN).

***In Situ* Hybridization**

Slides were taken from -80°C, fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), treated with 0.1 M triethanolamine, dehydrated in ascending ethanols and air-dried. Slides were incubated overnight at 62°C overlaid with 100 µl of hybridization buffer containing 1.5 million cpm/minute of

a 35-S labeled riboprobe prepared by reverse transcription using the Maxiscript kit (Ambion) according to the manufacturer's instructions. For control slides, antisense probe was replaced with 2 million cpm/minute of sense probe. Post-hybridization the slides were washed in descending concentrations of buffer, dehydrated in ascending ethanols and air-dried overnight. After imaging in a phosphorimager to confirm signal, slides were dipped in Kodak NTB emulsion, dried at 55°C, and then maintained in darkness at 4°C. After four weeks, emulsion was developed according to Kodak's recommendations, and sections were counterstained with toluidine blue before dehydration, clearing in xylene and coverslipping with Permount.

NADPH Diaphorase Histochemistry

Slides were taken from -80°C, air-dried briefly, and fixed for 20 minutes in ice-cold 0.1M phosphate buffer pH 7.4 (PB) containing 4% paraformaldehyde and1% glutaraldehyde. After rinsing well in PB, slides were incubated for two hours at 37°C in PB containing 0.3% triton-X, 0.1 mg/ml nitro blue tetrazolium, and 0.25 mg/ml β-NADPH. They were then rinsed, dehydrated, cleared with xylene and coverslipped with Permount.

Cell Counting and Statistical Analysis

Cells labeled by NADPH diaphorase staining or by *in situ* hybridization were counted with similar procedures using the Fractionator routine of the Stereo

Investigator software package (Microbrightfield, Williston, VT). A region of interest (shown in Fig. 6) was defined under low power, and then, under higher magnification, positive cells were counted that fell within 75 µm-square counting frames, placed on an 80 µm grid within the region. Cells expressing *nNOS* mRNA were clearly marked by dense clusters of silver grains after *in situ* hybridization, and were counted using a 40X objective. Cells stained by NADPH diaphorase histochemistry were much less distinct, and were counted using a 60X immersion objective. Slides were coded, and processed by an observer unaware of the treatment each animal had experienced.

Differences between the proportions of individuals displaying male-typical copulatory behavior in testosterone-treated and control groups were subjected to Fisher's Exact Test. Data from all three quantitative assays were analyzed using two-way ANOVA with time between implantation and sacrifice (TIME), and implant type (testosterone or blank: HORMONE) as independent variables and the experimentally determined value as the dependent variable. Planned comparisons between testosterone-treated and control groups at each time point were used to reveal the point at which the effect of HORMONE became significant.

RESULTS

Behavioral Reinstatement and nNOS Induction Have Similar Time-Courses

None of the blank-implanted individuals displayed any male-typical copulatory behavior except one individual on day 3. Among the testosterone-implanted individuals, proportions of individuals displaying mounting behavior increased with testosterone exposure (Fig. 7). Only for day 18 is the difference in behavior significantly different between testosterone-treated and control groups (Fisher's exact test, two-sided probability, $p<0.047$).

Measurements of *nNOS* mRNA abundance in laser microdissected fragments of PvPOA tissue are illustrated in Figure 9. The apparent trend is a steady increase in *nNOS* levels over time in testosterone-implanted animals, and no increase among blank-implanted animals. Two-way analysis of variance revealed significant main effects of HORMONE, with testosterone animals higher than controls ($F = 5.72$, $p<0.021$), and TIME ($F = 3.76$, $p<0.017$), while the interaction between HORMONE and TIME was marginal ($F =2.26$, $p<0.094$). The simple effect of HORMONE was examined with planned comparisons at each time point, and was significant only on day 18 (day 1, $p<0.701$; day 3, $p<0.259$; day 9, $p<0.224$; day 18, $p<0.005$).

Up-regulation of *nNOS* Expression is Observed in Periventricular Cell Population after Three days of Testosterone Exposure.

The probe derived from the whiptail *nNOS* clone recognized a single band at around 12 kilobases, a similar size to the dominant human *nNOS* transcript found in brain and other tissues (Park et al., 2000). After *in situ* hybridization using this probe, the distribution of *nNOS* expressing cells covered in silver grains matched well the previously reported distribution of NADPH diaphorase positive cells in the whiptail brain (Sanderson et al, 2006). Dense clusters of grains were visible even under low magnification in the striatum, the dorsal ventricular ridge, and the amygdaloid complex in all subjects regardless of treatment, while the septum and the cortex were almost devoid of signal. In the periventricular preoptic area (PvPOA), *nNOS* expression was dramatically increased by three or more days of testosterone exposure (Fig. 11). Expression of *nNOS* in this cell population was indistinguishable from blank-implanted animals after one day of testosterone exposure (Fig. 11A), but after 9 days was dramatically up-regulated (Fig. 11B). Cell counts are shown in Figure 11C. Subjecting these counts to two-way ANOVA revealed a Main Effect of HORMONE, ($p<0.0001$). Neither the effect of TIME or the interaction was significant ($p<0.067$; $p<0.019$, respectively). Planned comparisons between testosterone-treated and control groups at each

time point revealed that there was no difference after one day (p=0.482), but after three, nine or eighteen days, testosterone-treated animals had significantly more ($p < 0.005$, $p < 0.005$, $p < 0.039$, respectively). Numbers of clusters in the Dorsal Ventricular Ridge were counted and analyzed in a similar fashion (Fig. 13). There was no effect of HORMONE (p=0.135), there was a marginal effect of TIME ($p < 0.081$), and there was no significant interaction between the two factors (p=0.524).

NADPH diaphorase histochemistry yielded a distribution of stained cells similar to that identified by *in situ* hybridization, and to that previously described for NADPH diaphorase using perfusion-fixed tissue (Sanderson et al, 2006). In testosterone-exposed animals, lightly stained cell bodies were visible under high magnification in the periventricular preoptic area, mostly from 20µm to 100µm from the ventricle wall (Fig. 12B). In animals deprived of testosterone or exposed for only one day, these cells are scarcely detectable (Fig 12A). Numbers of these cells are displayed by group in Figure 12C. The pattern of up-regulation is similar to that revealed by *in situ* hybridization: expression increases with time in testosterone-exposed animals, but not in controls. Two-way ANOVA revealed significant effects of HORMONE ($p < 0.0001$) and TIME ($p < 0.01$), and a significant interaction between the two ($p < 0.038$). The simple effect of HORMONE was insignificant on day 1 (p=0.526), but significant at later time

points (day 3, $p<0.025$; day 9, $p<0.0006$; day 18, $p<0.001$). Numbers of cells in the Dorsal Ventricular Ridge were counted and analyzed in a similar fashion (Fig. 14). There was no effect of HORMONE ($p=0.506$), TIME ($p<0.847$), or significant interaction between the two factors ($p=0.797$).

DISCUSSION

The results described suggest unequivocally that nNOS in the whiptail preoptic area is up-regulated by testosterone, and further, that the time-course of up-regulation is comparable with the reinstatement of male-typical copulatory behavior. This is consistent with role proposed for the enzyme in the hormonal gating of the behavior.

Comparison of the time-courses of the various end-points is also informative. Both endpoints that relied on counting numbers of cells were significantly increased over controls within three days of testosterone exposure, and by nine days had reached their maximum values. On the other hand, levels of total nNOS mRNA, as measured by quantitative PCR, continued to increase robustly until the 18-day time point, when the highest level of copulatory behavior was also observed. A simple interpretation of these results is that a defined population of cells responds to testosterone stimulation by expressing nNOS. After three days' exposure, these cells are expressing at a high enough

level to become visible to the staining techniques used, and therefore countable. Thereafter, although expression in each cell continues to increase, as revealed by qPCR, the number of expressing cells does not.

This interpretation is consistent with the results of Sato et al. (2005), who found that ten days of testosterone exposure had no effect on the numbers of nNOS immunoreactive cells in the MPOA of castrated male rats, but did increase the optical density per cell. A similar effect was reported by Scordalakes et al. (2002), who examined the expression of nNOS in gonadectomized mice with various steroid receptor knockout genotypes, exposed to testosterone for several weeks. Wild-type mice and estrogen receptor knockouts had similar numbers of nNOS immunoreactive cells in the MPOA, but the area of immunoreactive tissue was greater in the wild-types, suggesting that testosterone (acting after aromatization, via the estrogen receptor) increases nNOS expression in each cell, but does not change the number of expressing cells.

The results of the present study are the opposite of those reported by Singh et al. (2000), with respect to the effect of androgens on *nNOS* expression. This discrepancy is probably best explained by anatomical differences: inasmuch as rostro-caudal positions can be compared between the rat and lizard preoptic areas, the tissue sample analyzed by Singh et al. (2000) was more caudal than the area examined in the present study. The target area of the present study was chosen on

the basis of a previous study suggesting that the expression of male-typical copulatory behavior was associated with nitric oxide synthesis in this location (Sanderson et al, 2006). The location of the tissue samples used by Singh et al. (2000) was selected to include the sexually dimorphic nucleus of the preoptic area (SDN-POA), on the basis of its established sexual dimorphism in size and its assumed role in controlling sexual behaviors. Not all neural structures involved in the control of a behavior are necessarily involved in the hormonal gating of that behavior, however, and the sexual dimorphism of the SDN-POA, a canonically *organizational* effect, does not necessarily strengthen its candidacy as an anatomical locus of behavioral *activation* by adult hormones. The absence of any robust effect of testosterone on the expression of nNOS in the DVR in the present study is further testament to the regional specificity of hormonal regulation.

Although evidence concerning the androgenic regulation of *nNOS* transcription in the preoptic area is limited, the phenomenon has been examined in other tissues. Several studies have established that androgens increase *nNOS* mRNA in the penis (e.g., Park et al., 1999). Sato et al. (2004) reported that in mice with genetic ablation of the androgen receptor (AR), the expression of *nNOS* mRNA was reduced in a tissue sample described as “hypothalamus”. On the other hand, Scordalakes et al. (2002) found no difference in preoptic nNOS immunoreactivity between wild type mice and mice with a loss of function

mutation in the AR, a difference that is presumably due to the different anatomical locations from which the samples were derived.

Considerable attention has been devoted to the role of preoptic nNOS expression in the regulation of gonadotropin releasing hormone (GnRH) secretion in females. Lamar et al. (1999) reported that *nNOS* mRNA, protein and activity all rose in the POA but not medial-basal hypothalamus on proestrus afternoon in cycling female rats. Ishihara et al. (2002), using *in situ* hybridization, examined the response of male and female prepubertal rats to a combination of estradiol and progesterone that in the females evoked a large surge of luteinizing hormone (LH). In the rostral POA there were more *nNOS* mRNA expressing cells in gonadectomized females than in gonadectomized males, but estradiol and progesterone suppressed this index in females to below the level of males, who were unaffected by the hormones. Thus, although the facilitatory influence of NO on GnRH release is well established (reviewed by McCann et al., 2003), the population of nitrergic cells involved and their responses to gonadal steroids are still under investigation (discussed by Ishihara et al., 2002).

The area of the brain labeled "preoptic" thus appears to include various populations of cells in which nNOS expression is suppressed by androgen (Singh et al, 2000), increased by androgen (Scordalakes et al., 2002, and references therein; present study), suppressed by ovarian hormones associated with the LH

surge (Ishihara et al., 2002) and increased by ovarian hormones associated with the LH surge (Lamar et al., 1999). This anatomical variability was examined explicitly by Ishihara et al. (2002), who reported that the effect of ovarian hormones on *nNOS* expression was detectable in sections 60 or 120 microns caudal to the organum vasculosum of the lamina terminalis (OVLT), but not at the level of the OVLT itself, or in sections 180 microns caudal to the OVLT. These observations are perhaps not surprising in view of the numerous neural and behavioral processes in which preoptic nitric oxide synthesis has been implicated. These include the control of sleep (Stenberg, 2007) and maternal behaviors including pup retrieval(Service and Woodside, 2007) and aggression (Gammie and Nelson, 2000), in addition to the control of sexual behavior and gonadotropin release already discussed.

The possible existence of multiple nitrergic systems within the POA would imply that traditionally dissected tissue samples, such as those used by Singh et al. (2000) and Lamar et al. (1999) may include nNOS-expressing cells from more than one population, potentially with disparate responses to steroids. In the whiptail, the population of nitrergic cells putatively involved in hormonal gating of male copulatory behavior is limited to a small periventricular area with a rostro-caudal extent of only a few hundred microns (Sanderson et al., 2006). Methods based on cell counting, such as *in situ* hybridization or

immunohistochemistry, enable the precise localization of cells being assayed and may be more appropriate for the anatomically heterogeneous hypothalamus/preoptic area. On the other hand, quantitative differences that are hard to measure precisely with these techniques may also be important. For example, the difference between the two-fold up-regulation of *nNOS* revealed by qPCR after nine days' testosterone exposure in this study, and the five fold up-regulation seen after eighteen days' exposure appears to be important behaviorally, since the latter was associated with a significantly higher proportion of individuals copulating, perhaps because nNOS expression must reach a certain threshold in order to enable behavior. In this and similar situations requiring the combination of anatomical precision and reliable quantitative measurements over a wide range of expression levels, the combination of laser microdissection and quantitative PCR promises to be a valuable tool.

Chapter Five: Conclusion

The experiments described in this Dissertation were conducted to test a number of key predictions of a hypothetical model elaborated by Hull et al. (2004) describing the cellular mechanisms underlying androgenic gating of male-typical copulatory behavior.

The first of these predictions, that pharmacological inhibition of nitric oxide synthesis ought to suppress male-typical copulatory behavior has been extensively examined in rats, (e.g., Lagoda et al, 2004), and the experiment described in Chapter Two was conducted principally to establish that the involvement of nitric oxide in hormonal gating in whiptails is comparable to the phenomenon seen in rats. Its conclusion, that the manipulation has the expected suppressive effect, is consistent with the model, but subject to the caveat that the behavioral effects of systemic drug administration are likely to be mediated by several anatomical loci, and not restricted to the POA that is the focus of this investigation. This lack of anatomical specificity raises the question of why more general effects of the drug are not observed. Del Bel et al. (2005) discuss this issue with respect to studies of the anxiolytic effects of NOS inhibitors, and suggest that the inverted U shaped response curve often reported may well be an

artifact generated by the combination of genuine anxiolysis at low doses, and motor deficits associated with abnormal striatal neurotransmitter turnover at high doses. Chapter Two of this Dissertation includes a description of how a dose of L-NAME was chosen that suppressed copulatory behavior without detectable effects on motor function. Presumably even at this dose, neural systems affected by lower doses, such as those discussed by Del Bel et al (2005) may have been affected, but the behavioral test employed was not designed to detect such effects.

The second prediction, that preoptic nitric oxide synthesis would be higher in animals expressing male-typical copulatory behavior was tested using a surrogate marker for nitric oxide synthesis, citrulline immunoreactivity. While the results of this experiment did suggest that testosterone-treated animals given an opportunity to copulate had higher levels of synthesis than testosterone-deprived animals, it was not possible to determine whether this effect was a result of the expression of the behavior, or simply a consequence of the testosterone exposure. Citrulline immunoreactiviy in testosterone-treated animals given no opportunity to copulate was intermediate between testosterone-deprived animals and testosterone-treated animals who copulated, and not clearly statistically different from either. Since enhanced NO synthesis following exposure to a receptive female is a clear prediction of the model, this experiment ought to be repeated

with an adequate number of animals to distinguish between these experimental groups.

The third prediction, that preoptic NOS abundance would be greater in testosterone-exposed animals, was tested with respect to mRNA and protein. Protein was inferred from NADPH diaphorase staining, while mRNA abundance was assessed by quantitative PCR as well as by radioactive *in situ* hybridization histochemistry. Protein expression was first examined in testosterone-treated females, as described in Chapter Three. Numbers of NAPDH diaphorase positive cells in the periventricular preoptic area were dramatically increased by a long period of testosterone exposure that induced robust male-typical copulatory behavior in these animals. The greatest weakness of this experiment is that while NAPDH diaphorase histochemistry is considered a reliable marker of NOS-expressing cells (Dawson et al., 1991), it is not an infallible marker of the single gene product nNOS, that is the central player in the model described by Hull et al. (2004).

The cloning of the whiptail nNOS gene and measurement of mRNA expression resolved this problem. The identity of the gene is clearly established by sequence homology and the unusually large transcript size, as described in Chapter Four, and the fact that nNOS mRNA expression shows a similar response to testosterone exposure as does NADPH diaphorase expression, particularly in its

spatial distribution, is strong evidence that both paradigms are assessing expression of the same gene product.

Simplistically, the androgen sensitive switch at the core of the proposed gating mechanism is assumed to have two critical characteristics:

1. It should be necessary and sufficient for the expression of copulatory behavior, i.e., its presence in the preoptic area ought to enable copulatory behavior in the absence of testosterone, and its absence should eliminate behavior even in the presence of testosterone.
2. It should be strongly regulated by testosterone, i.e., present after testosterone exposure and absent after testosterone deprivation.

The results of the experiments suggest that nNOS fulfils the second of these criteria better than any other gene yet investigated in this context; its expression in the periventricular preoptic area is dramatically increased by testosterone in exactly the temporal pattern than would be predicted for a testosterone-sensitive switch. On the other hand, the pharmacological suppression of NOS described in Chapter 2, as well as similar experiments performed with rats (e.g., Lagoda et al., 2004) suggest that nNOS does not meet the first of these criteria. The effects of pharmacological inhibition of NO synthesis have only a partial effect on male copulatory behavior, eliminating copulation in some individuals, retarding it in some, and having no effect on

others. In other words, although NO has a facilitatory effect on the expression of male typical copulatory behavior, this effect can be achieved by other, NO-independent mechanisms. It seems likely, therefore, that rather than being a simple and omnipotent switch, nNOS acts as one influence on a neural network that determines the probability of a copulatory response based on several such influences. The contribution of nNOS is testosterone-dependent and positive, while other influences might be either positive or negative, and might or might not be hormone-dependent. Such a model would explain the subtle effects reported for experimental manipulations of various neurotransmitters in the preoptic area that are discussed in the Introduction, and is consistent with the known influence of factors such as sexual experience, nutritional status, and stress on the expression of copulatory behavior (e.g., Coolen, 2004; Santos et al., 2004; Retana-Marquez et al., 1996).

This more sophisticated model of nNOS' role in androgenic gating of male-typical copulatory is still consistent with the hypothesis that progesterone acts via nNOS to activate male-like copulatory behavior in parthenogenic whiptails. The prediction would be that in the periovulatory phase of the ovarian cycle, when progesterone levels are high and male-like copulatory behavior is expressed, nNOS expression in the PvPOA would be higher than during vitellogenesis, when male-like copulatory behavior is not observed.

Figures

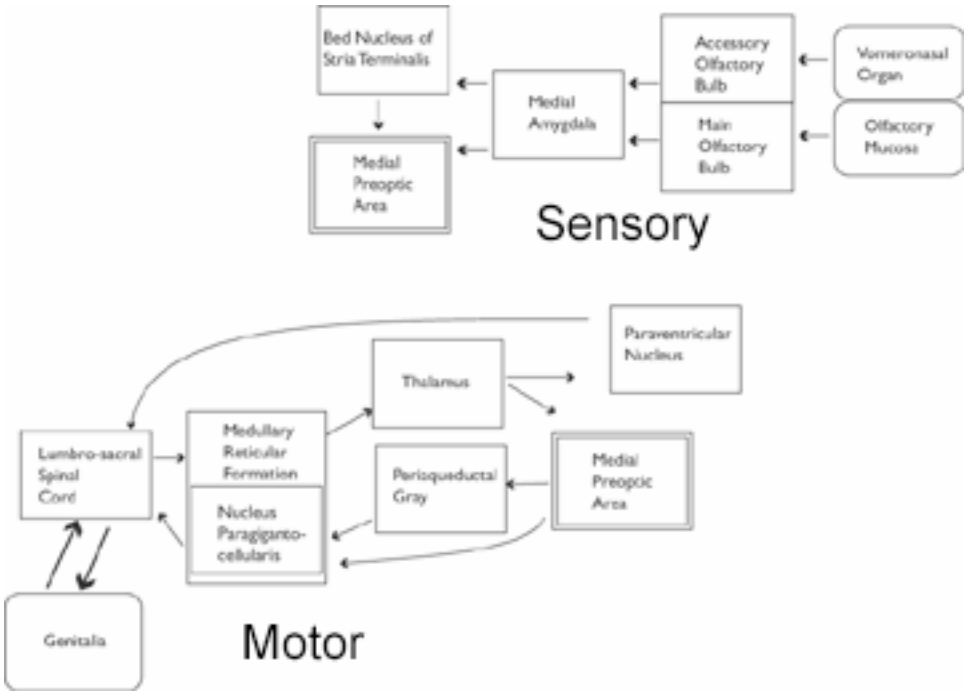

Figure 1. Schematic representation of brain areas involved in mediating male-typical copulatory behavior in rodents. The top half of the figure illustrates areas in which sensory information is processed in a largely hormone-independent way. In particular, olfactory cues are detected in the olfactory mucosa, and reach the medial amygdala via the main olfactory bulb, while pheromonal cues are detected by the vomeronasal organ and reach the medial amygdala via the accessory olfactory bulb. The bottom half of the figure represents brain areas involved in mediating the motor behaviors of copulation, which are essentially a reflex circuit involving integration of the movements of mounting, intromission and thrusting by brainstem motor nuclei such as the nucleus paragigantocellularis, using sensory feedback from the genitals and surrounding areas. The medial preoptic area is thought to be a hormonal "gate" controlling the nature of the information flow from the sensory module to the motor module

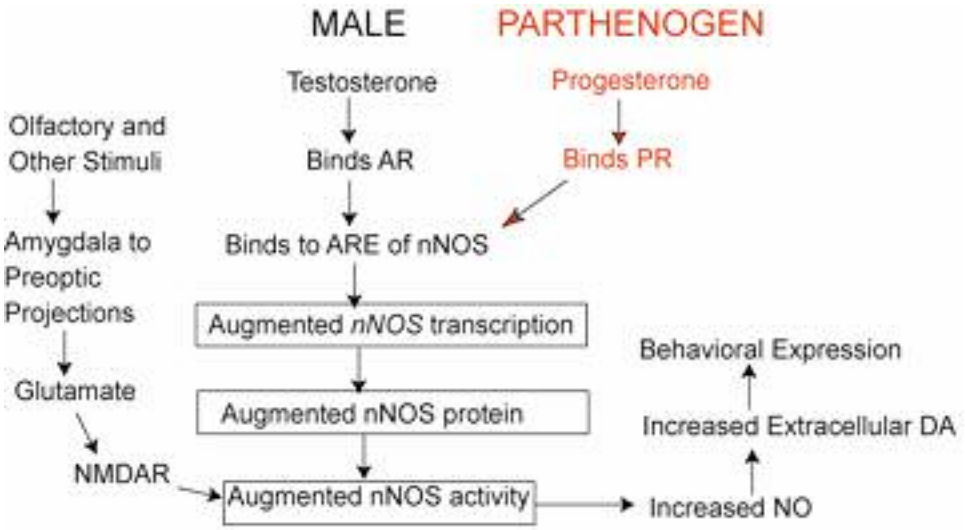

Figure 2. Hypothetical model of neural and molecular mechanisms underlying gating of male-typical copulatory behavior by testosterone or progesterone. Sensory information encoding the presence of a receptive female reaches the preoptic area in the form of glutamatergic inputs that stimulate NMDA receptors on preoptic neurons. In a hormone-deprived individual, this glutamatergic stimulation does not result in the expression of copulatory behavior. Testosterone exposure causes the expression of nNOS, which, when activated by calcium influx through the NMDA receptor, synthesizes NO. The NO thus produced acts to increase extracellular dopamine levels, possibly by inhibiting the dopamine re-uptake transporter, and dopamine changes the properties of preoptic neurons so that now the same stimulus provokes a copulatory response. Boxes highlight components of this model that were explicitly tested by experiments described in this dissertation. An interesting possibility not tested is that the exhibition of male-like copulatory behavior by morphologically female whiptails of the parthenogenic species C. uniparens represents crosstalk between this pathway and progesterone signaling (represented in red).

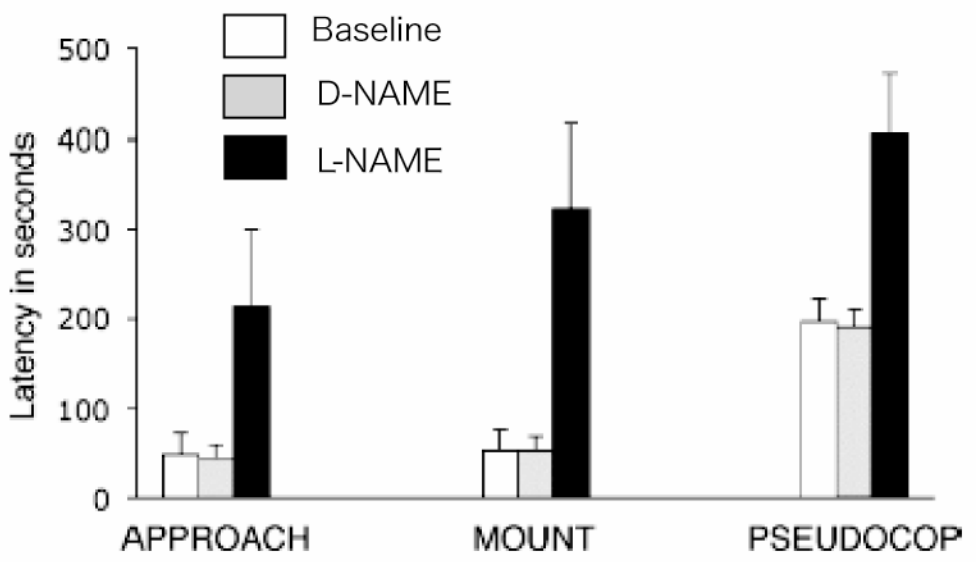

Figure 3. Effect of nitric oxide synthase inhibition on whiptail copulatory behavior. Bars show mean latencies to exhibit copulatory behaviors for animals in baseline (white bars), D-NAME (inactive isomer of L-NAME, gray bars), and L-NAME (nitric oxide synthase inhibitor, black bars) conditions. Small bars show standard errors. Animals that failed to show a behavior were assigned the maximum latency of 600 s. For statistical results see text.

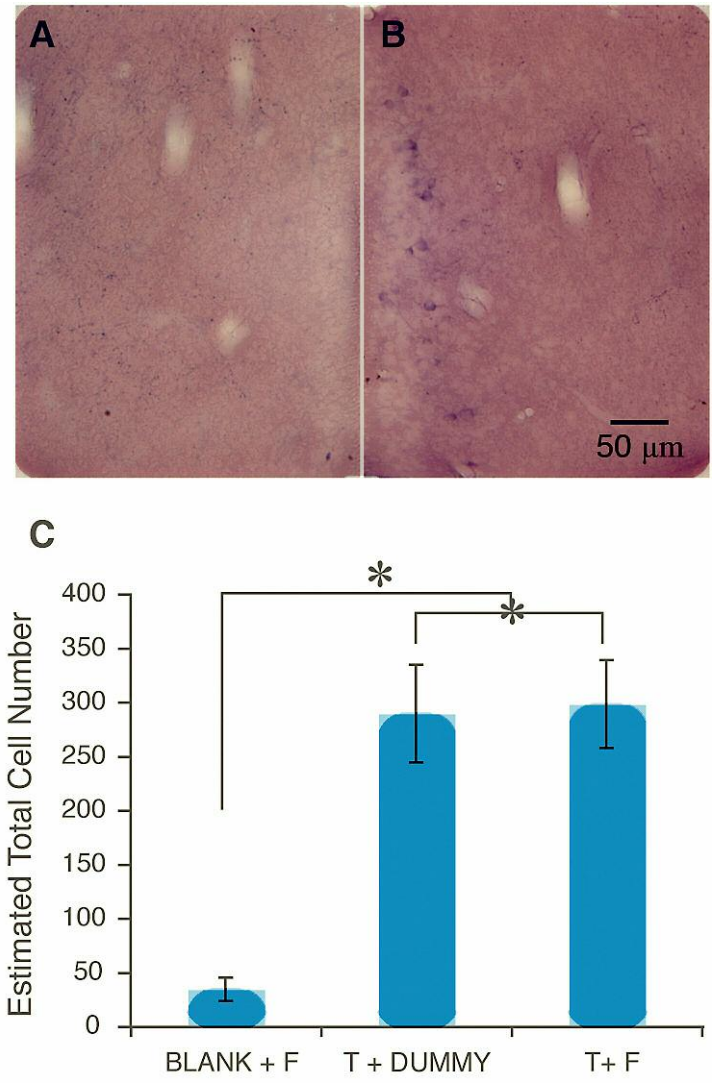

Figure 4. The effect of testosterone (T) treatment on NADPH diaphorase-positive cell numbers in the periventricular preoptic area of ovariectomized female whiptail lizards (Cnemidophorus inornatus). Cells are rarely observed in ovariectomized animals given blank (BLANK) Silastic capsules (A), but can clearly be seen close to the third ventricle in an ovariectomized female given a Silastic capsule containing T (B). Figure 4(C) shows estimated total numbers of these periventricular NADPH-d cells in the three experimental groups. The asterisk signifies that the two T-treated groups (T-treated and exposed to a dummy, or t-treated and allowed to copulate with a receptive female {F}) are significantly higher than the BLANK-implanted group ($p = 0.0002$)

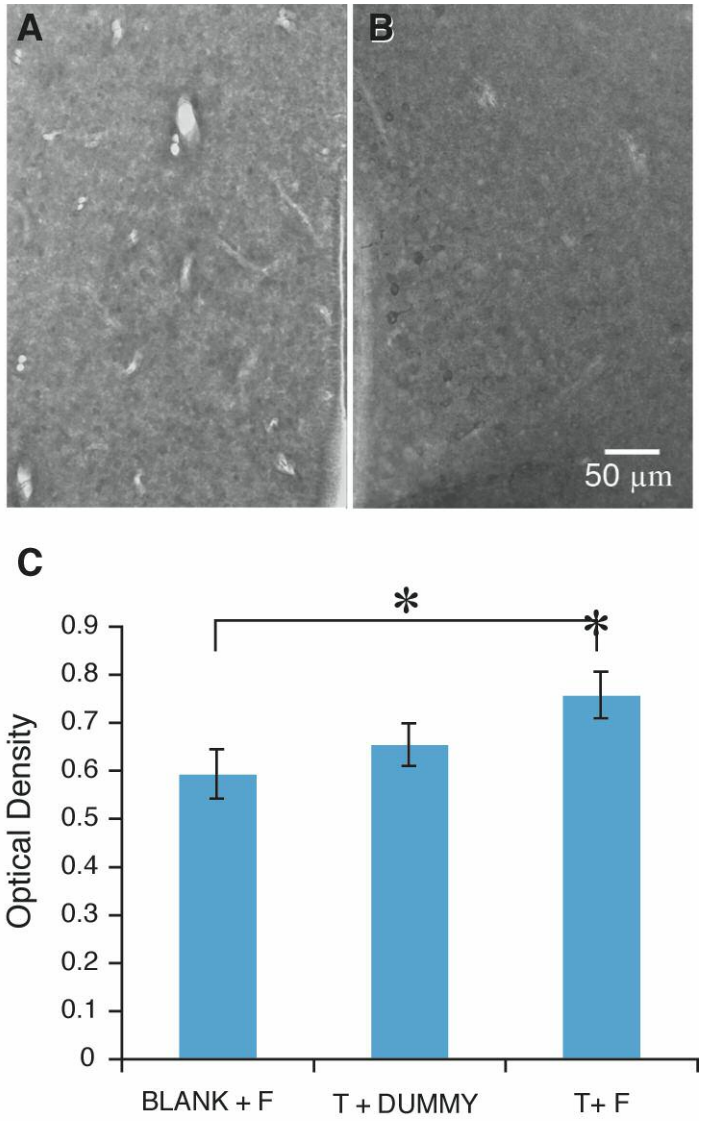

Figure 5. The effect of testosterone (T) treatment and copulatory behavior on citrulline immunoreactivity in the preoptic area of ovariectomized female whiptail lizards. (A) shows a coronal section through the brain of an individual implanted with a blank (BLANK) Silastic capsule, and (B) an individual given a Silastic capsule containing T and exhibiting male-typical copulatory behavior toward a receptive stimulus female {F} immediately before sacrifice. Micrographs (A) and (B) were captured under similar lighting conditions and thenceforth processed identically. The neuropil is generally darker, suggesting greater citrulline immunoreactivity and more nitric oxide synthesis in the animal having exhibited male-typical copulatory behavior (B). A few darkly stained cells are visible close to the third ventricle. Optical densities measured over this area are compared between groups in (C). The asterisk signifies that the T+F group is significantly higher than the BLANK-implanted group ($p = 0.011$).

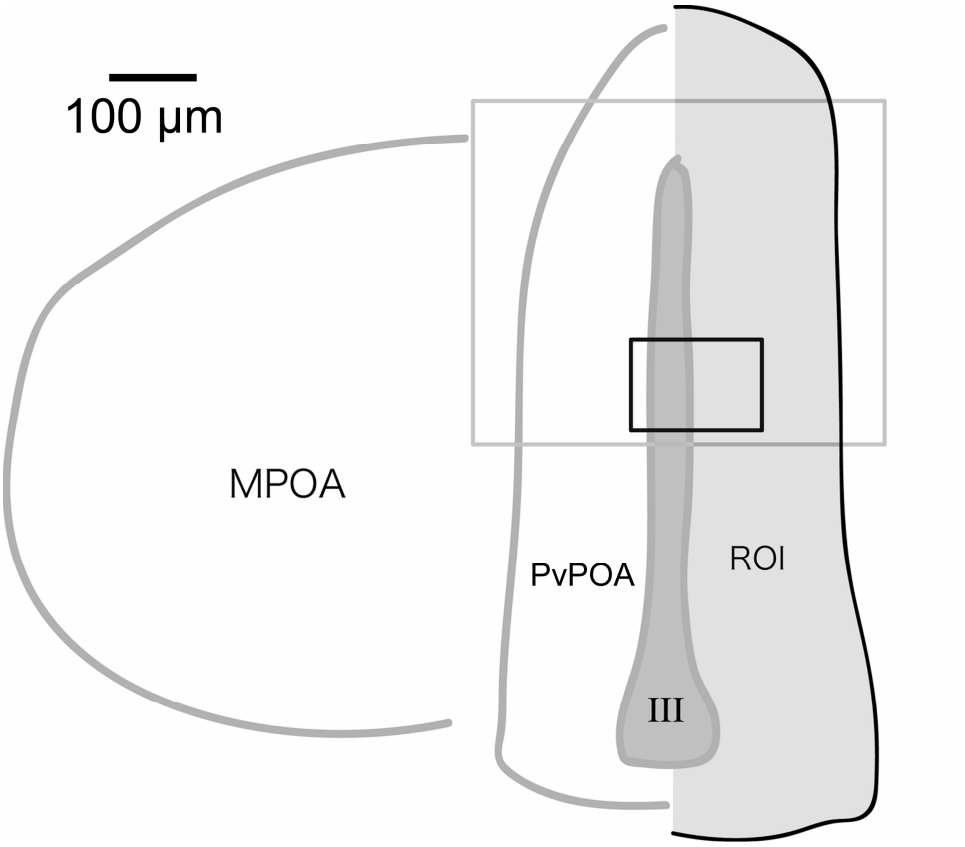

Figure 6. Schematic representation of coronal section through whiptail preoptic area showing various regions of interest, including the periventricular preoptic area (PvPOA) adjacent to the third ventricle (III), and the more lateral medial preoptic area (MPOA). The shaded area on the right represents the region of interest (ROI) from which tissue for qPCR was captured, and in which cells were counted. The light and dark boxes represent the area of the micrographs in Figure 11 and Figure 12, respectively.

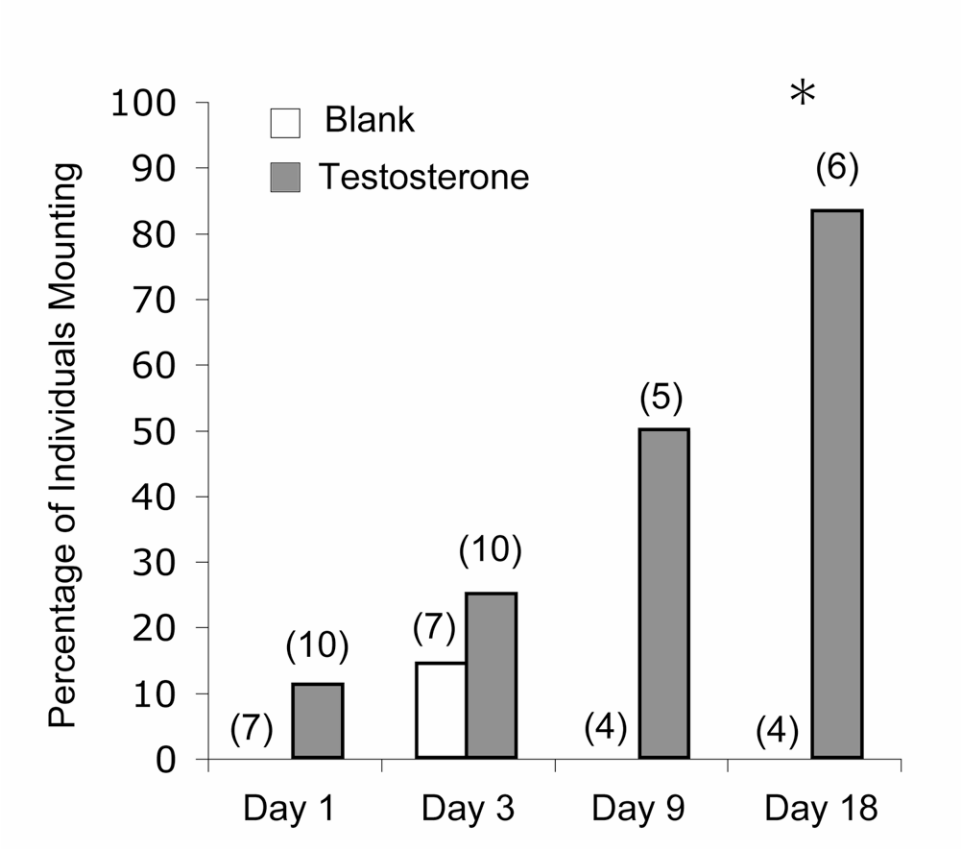

Figure 7. Percentage of individuals exhibiting male-typical copulatory behavior after various lengths of time following implantation of testosterone-filled (gray bars) or blank (empty bars) Silastic capsules. Numbers in parentheses above bars are group sizes. (* $p<0.05$).

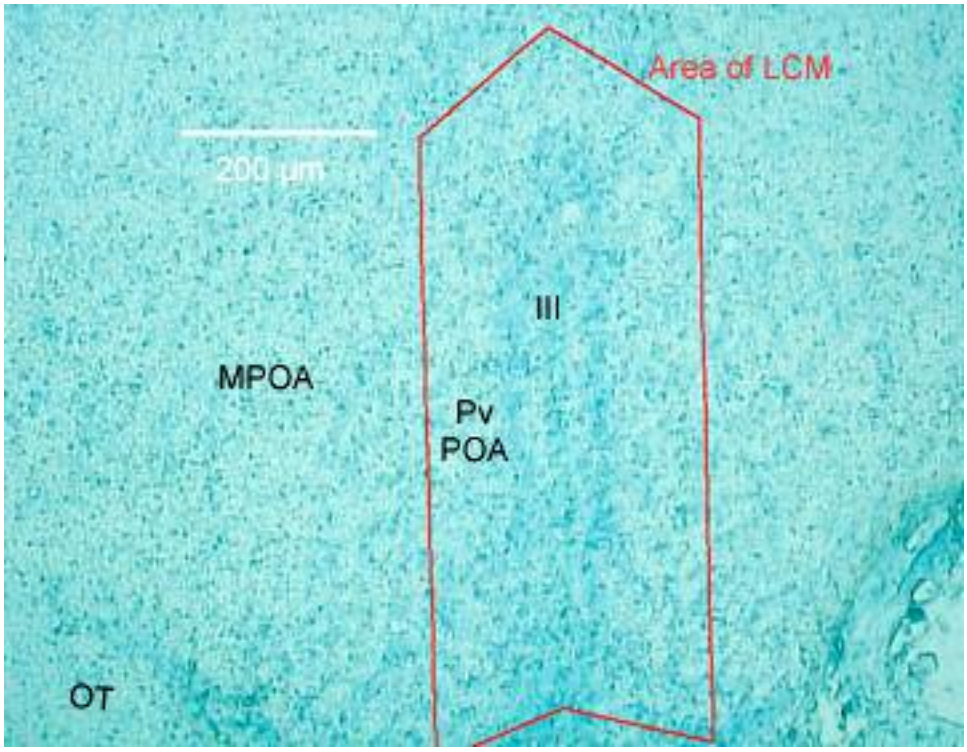

Figure 8. Toluidine blue stained section at the level of the preoptic area prior to laser capture microdissection. The third ventricle (III) is faintly delineated by darker staining cells, and the optic tract (OT) can be made out in the two bottom corners. The area outlined in red including the periventricular preoptic area (PvPOA) is the fragment excised by the laser, and RNA from tissue contained within the outline was extracted and reverse transcribed for measurements of nNOS and 18S transcript levels.

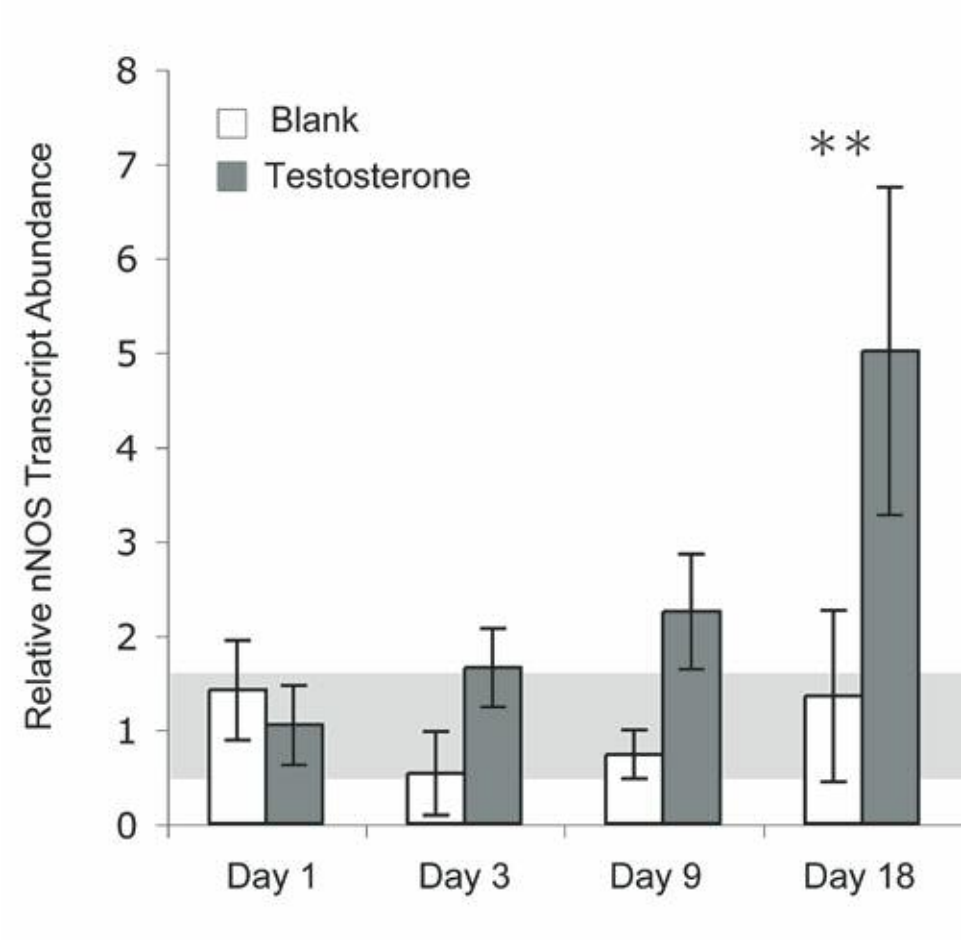

Figure 9. Relative nNOS transcript abundance in laser microdissected fragments of PvPOA tissue of same animals as in Figure 7. Raw measures of nNOS transcript abundance are normalized relative to 18S ribosomal RNA abundance and calibrated to the mean value of the blank-implanted controls to yield fold-change over baseline (so that the average individual among all controls would have a value of 1). Error bars show SE, and the 95% confidence interval around the global mean of the blank-treated animals is shown as a light gray band. Asterisks indicate significant difference between testosterone and blank groups at each time point (** $p<0.005$).

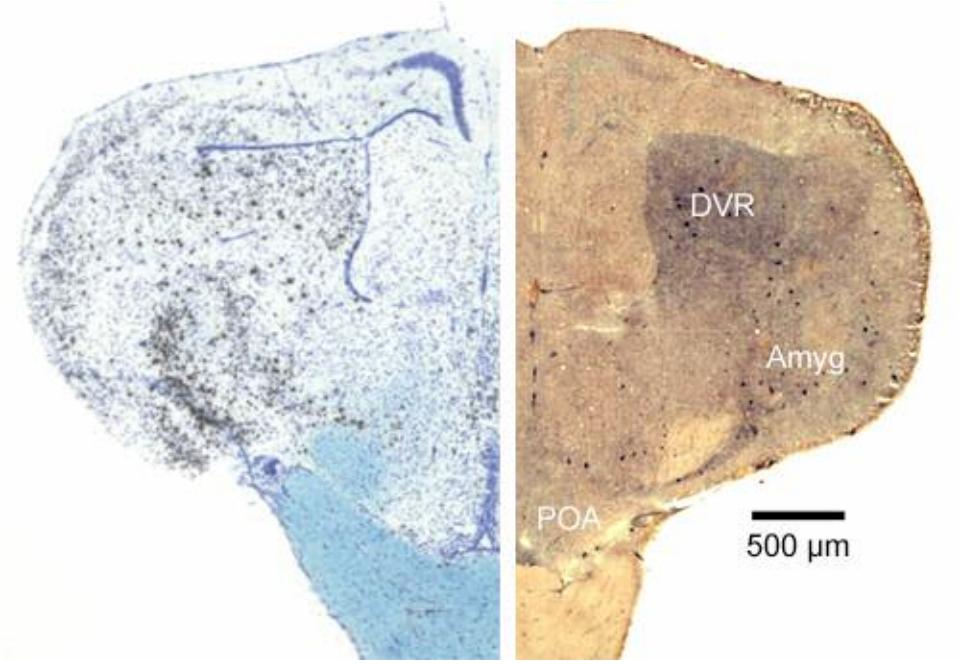

Figure 10. Half coronal sections at the level of the preoptic area showing nNOS ISH and NADPH diaphorase. Both techniques reveal nNOS expression in the Dorsal Ventricular Ridge (DVR) and Amygdala (Amyg). Signal in the preoptic area (POA) is fainter and can be observed best at higher magnification – see Figures 11 and 12.

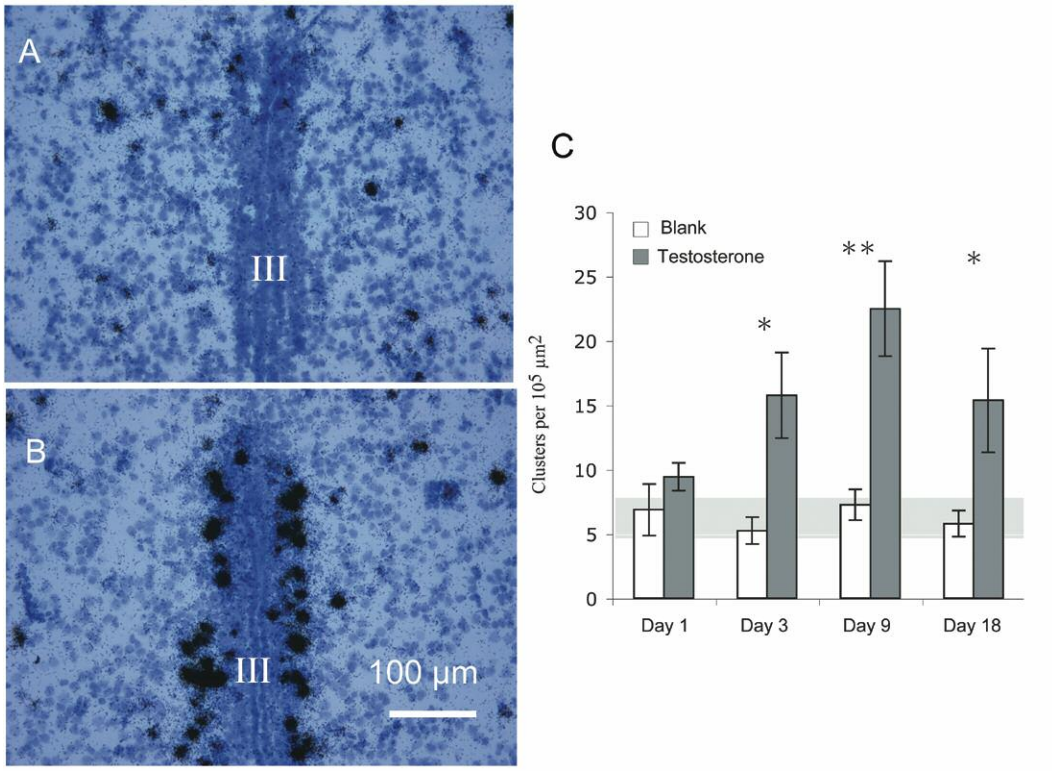

Figure 11. Expression of *nNOS* in the periventricular preoptic area (PvPOA) after various periods of testosterone exposure, as revealed by *in situ* hybridization. (A) Coronal section through the rostral preoptic area (see Fig. 5, light gray box) of an animal exposed to testosterone for one day, showing dorsal extremity of the third ventricle (III). Scattered, lightly-expressing cells are marked by small clusters of silver grains. (B) Similar micrograph from an animal exposed to testosterone for nine days. A population of cells close to the third ventricle shows dramatically increased *nNOS* expression. (C) Histogram showing numbers of *nNOS*-expressing cells inferred from silver grain clusters in the PP. Asterisks indicate significant difference in cell number between testosterone and blank group at each time point (*$p<0.05$, **$p<0.005$). For comparison, the 95% confidence interval around the global mean of the blank-treated animals is shown as a light gray band.

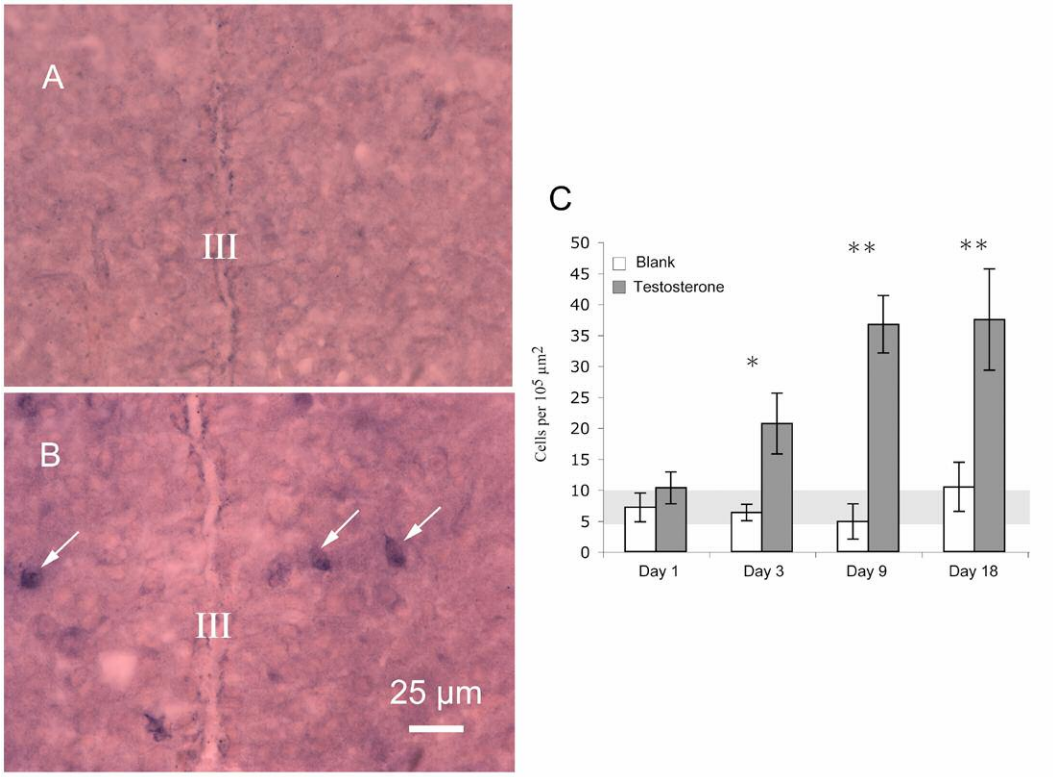

Figure 12. Effect of various periods of testosterone exposure on NADPH diaphorase positive cell numbers in the whiptail periventricular preoptic area. (A) High power photomicrograph of area (see Fig. 5, dark gray box) adjacent to third ventricle (III) of a 1-day testosterone-exposed animal showing few NADPHd positive cells. (B) similar micrograph from an animal after 18 days testosterone exposure, showing NADPHd positive cells (white arrows). (C) Histogram of numbers of NADPHd + cells in the periventricular preoptic areas of animals implanted with testosterone-filled (gray bars) or blank (empty bars) Silastic capsules for various lengths of time before sacrifice. Asterisks indicate significant difference in cell number between testosterone and blank group at each time point (*$p<0.01$, **$p<0.001$). For comparison, the 95% confidence interval around the global mean of the blank treated animals is shown as a light gray band.

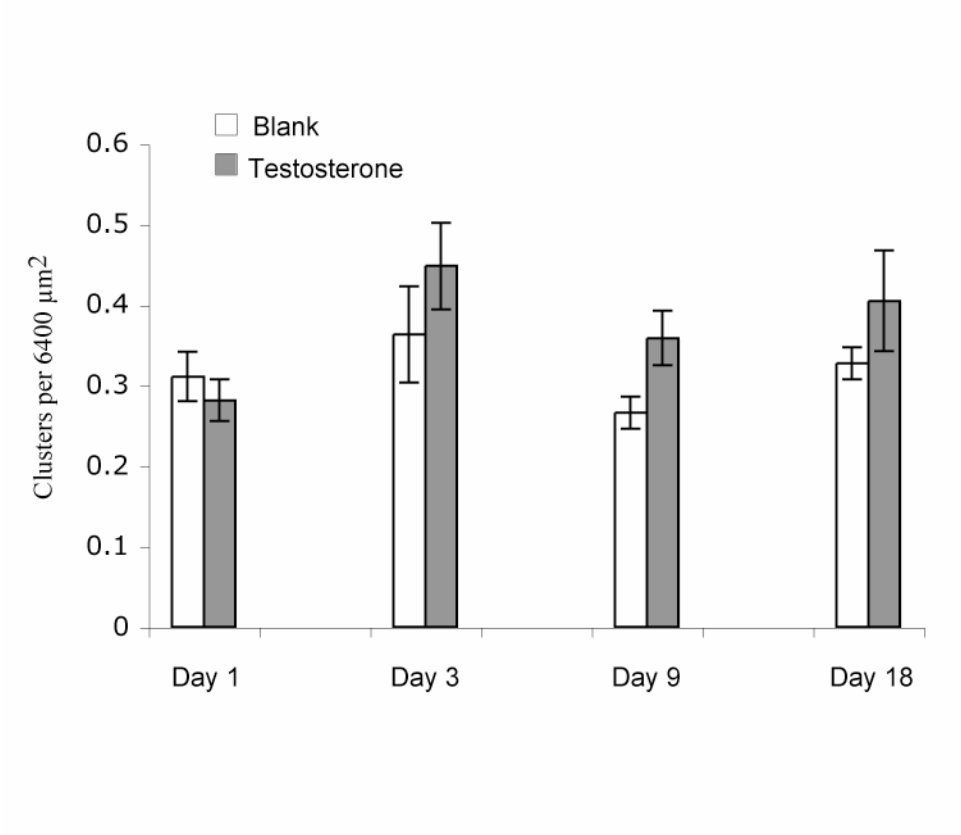

Figure 13. Effect of various periods of testosterone exposure on nNOS mRNA abundance in the whiptail DVR – *in situ* hybridization. Vertical axis represents the number of clusters of silver grains per 64000 μm^2 counted on autoradiograph – white bars represent control animals implanted with blank Silastic implants; gray bars represent animals implanted with testosterone-filled capsules.

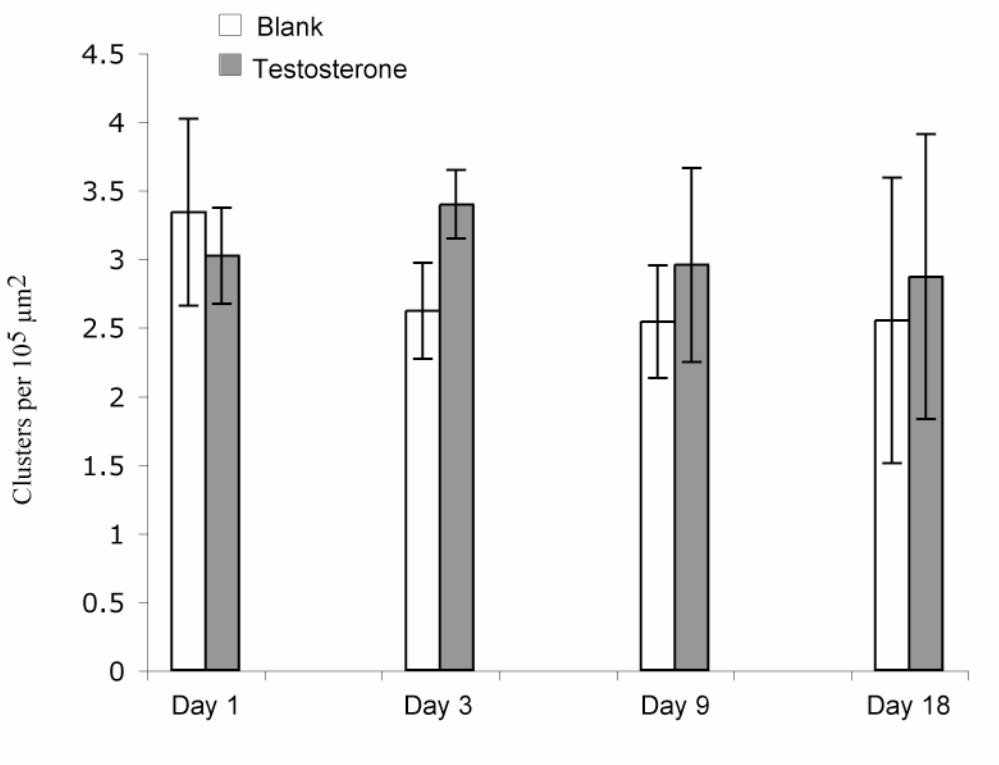

Figure 14. Effect of various periods of testosterone exposure on nNOS expression as revealed by NADPH diaphorase histochemistry in the DVR. Vertical axis represents the number of stained cells per 100,000 μm^2 – white bars represent control animals implanted with blank Silastic implants; gray bars represent animals implanted with testosterone-filled capsules.

Bibliography

Argiolas A. (1994). Nitric oxide is a central mediator of penile erection. Neuropharmacology 33 1339–44.

Ahern GP, Klyachko VA, Jackson MB. (2002). cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. Trends Neurosci. 25 510-7

Alsum P, Goy RW. (1974). Actions of esters of testosterone, dihydrotestosterone, or estradiol on sexual behavior in castrated male guinea pigs. Horm Behav. 5 207-17.

Bachir LK, Garrel G, Lozach A, Laverriere JN, Counis R. (2003). The rat pituitary promoter of the neuronal nitric oxide synthase gene contains an Sp1-, LIM homeodomain-dependent enhancer and a distinct bipartite gonadotropin-releasing hormone-responsive region. Endocrinology. 144 3995-4007.

Balthazart J, Baillien M, Charlier TD, Cornil CA, Ball GF. (2003). The neuroendocrinology of reproductive behavior in Japanese quail. Domest Anim Endocrinol. 25 69-82.

Balthazart J, Sante P, Ball GF. (1991). Testosterone effects on staining density and autoradiographic investigations of the alpha 2-adrenergic receptor in the medial preoptic nucleus of the Japanese quail: relationship to the activation of reproductive behavior. Arch Int Physiol Biochim Biophys. 99 385-92.

Benelli A, Bertolini A, Poggioli R, Cavazzuti E, Calza L, Giardino L, Arletti R. (1995). Nitric oxide is involved in male sexual behavior of rats. Eur J Pharmacol. 294 505-10.

Bialy M, Beck J, Abramczyk P, Trzebski A, Przybylski J. (1996). Sexual behavior in male rats after nitric oxide synthesis inhibition, Physiol. Behav. 60 139–143.

Bordieri L, Persichini T, Venturini G, Cioni C. (2003). Expression of nitric oxide synthase in the preoptic-hypothalamo- hypophyseal system of the teleost Oreochromis niloticus. Brain Behav Evol 62 43–55.

Brann DW, Zamorano PL, Chorich LP, Mahesh VB. (1993). Steroid hormone effects on NMDA receptor binding and NMDA receptor mRNA levels in the hypothalamus and cerebral cortex of the adult rat. Neuroendocrinology. 58 666-72.

Cartledge J, Minhas S, Eardley I. (2001). The role of nitric oxide in penile erection, Expert Opin. Pharmacother. 2 95–107.

Castagna C, Obole A, Viglietti-Panzica C, Balthazart J, Panzica GC. (1999). Effects of testosterone on the synaptology of the medial preoptic nucleus of male Japanese quail.
Brain Res Bull. 50 241-9.

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics. 21 2933-42.

Chiavegatto S, Nelson RJ. (2003). Interaction of nitric oxide and serotonin in aggressive behavior. Horm Behav 44 233–241

Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, Lipton SA. (2000). Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. Nat Neurosci. 3 15-21.

Clark JT, Smith ER, Davidson JM. (1985). Testosterone is not required for the enhancement of sexual motivation by yohimbine. Physiol. Behav. 35 517–521.

Cooke BM, Tabibnia G, Breedlove SM. (1999). A brain sexual dimorphism controlled by adult circulating androgens. Proc Natl Acad Sci U S A. 96 7538-40.

Coolen LM. (2004). Involvement of nitric oxide in sexual learning via action in the medial preoptic area: theoretical comment on Lagoda et al. (2004). Behav Neurosci. 118 1473-5.

Cornil CA, Balthazart J, Motte P, Massotte L, Seutin V. (2002). Dopamine activates noradrenergic receptors in the preoptic area. J Neurosci. 22 9320-30.

Crews D. (2005). Evolution of neuroendocrine mechanisms that regulate sexual behavior. Trends Endocrinol Metab. 16 354-61.

Crews D, Fitzgerald KT. (1980). Sexual behavior in parthenogenetic lizards (*Cnemidophorus*). Proc Natl Acad Sci USA 77 499–502.

Crews D, Gustafson JE, Tokarz RR. (1983). Psychobiology of parthenogenesis. In: Huey RB, Pianka ER, Schoener TW, editors. Lizard ecology. Studies of a model organism. Cambridge: Harvard University Press, p 205–231.

Cuthbertson S, Zagvazdin YS, Kimble TD, Lamoreaux WJ, Jackson BS, Fitzgerald ME, Reiner A. (1999). Preganglionic endings from nucleus of Edinger-Westphal in pigeon ciliary ganglion contain neuronal nitric oxide synthase. Vis Neurosci 16 819–834.

Davidson JM. (1966). Characteristics of sex behaviour in male rats following castration. Anim Behav. 14 266-72.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88 7797–7801.

Dominguez JM, Gil M, Hull EM. (2006). Preoptic glutamate facilitates male sexual behavior. J Neurosci. 26 1699-703.

Dominguez JM, Hull EM. (2005). Dopamine, the medial preoptic area, and male sexual behavior. Physiol Behav 86:356–368.

Dominguez J, Riolo JV, Xu Z, Hull EM. (2001). Regulation by the medial amygdala of copulation and medial preoptic dopamine release. J Neurosci 21 349–355.

Du J, Hull EM. (1999) Effects of testosterone on neuronal nitric oxide synthase and tyrosine hydroxylase. Brain Res. 836 90-8.

Fernandez-Guasti A, Escalante AL, Ahlenius S, Hillegaart V, Larsson K. (1992). Stimulation of 5-HT1A and 5-HT1B receptors in brain regions and its effects on male rat sexual behaviour. Eur J Pharmacol. 210 121-9.

Fernandez-Guasti A, Larsson K, Beyer C. (1986a). Effect of bicuculline on sexual activity in castrated male rats. Physiol Behav. 36 235-7.

Fernandez-Guasti A, Larsson K, Beyer C. (1986b). GABAergic control of masculine sexual behavior. Pharmacol Biochem Behav. 24 1065-70.

Gabriel SM, Clark JT, Kalra PS, Kalra SP, Simpkins JW. (1988). Chronic morphine and testosterone treatment: effects on norepinephrine and serotonin metabolism and gonadotropin secretion in male rats. Brain Res. 447 200-3.

Hadeishi Y, Wood RI. (1996). Nitric oxide synthase in mating behavior circuitry of male Syrian hamster brain. J Neurobiol 30 480–492.

Hanbauer I, Wink D, Osawa Y, Edelman GM, Gally JA. (1992). Role of nitric oxide in NMDA-evoked release of [3H]-dopamine from striatal slices. Neuroreport. 3 409-12.

Hull EM, Bitran D, Pehek EA, Warner RK, Band LC, Holmes GM. (1986). Dopaminergic control of male sex behavior in rats: effects of an intracerebrally-infused agonist. Brain Res. 370 73-81.

Hull EM, Dominguez JM (2006). Getting his act together: roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. Brain Res 1126 66-75

Hull EM, Du J, Lorrain DS, Matuszewich L. (1995). Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. J Neurosci. 15 7465-71.

Hull EM, Meisel RL, Sachs BD. (2002). Male sexual behavior. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT (Editors) Horm. Brain Behav. vol. 1, Elsevier, Amsterdam pp. 1–138.

Hull EM, Muschamp JW, Sato S. (2004). Dopamine and serotonin: influences on male sexual behavior. Physiol Behav. 83 291-307.

Ishihara T, Orikasa C, Araki T, Sakuma Y. (2002). Sex difference in the expression and regulation of nitric oxide synthase gene in the rat preoptic area. Neurosci Res. 43 147-54.

Jeong Y, Won J, Kim C, Yim J. (2000). 5'-Flanking sequence and promoter activity of the rabbit neuronal nitric oxide synthase (nNOS) gene. Mol Cells. 10 566-74.

Kawata M. (1995). Roles of steroid hormones and their receptors in structural organization in the nervous system. Neuroscience Research 24 1-46.

Keilhoff G, Reiser M, Stanarius A, Aoki E, Wolf G. (2000). Citrulline immunohistochemistry for demonstration of NOS activity in vivo and in vitro. Nitric Oxide 4 343–53.

Kiss JP, Vizi ES. (2001). Nitric oxide: a novel link between synaptic and nonsynaptic transmission. Trends Neurosci 24 211–15.

Lagoda G, Muschamp JW, Vigdorchikk A, Hull EM. (2004). A nitric oxide synthase inhibitor in the medial preoptic area inhibits copulation and stimulus sensitization in male rats, Behav. Neurosci. 118 1317–1323.

Lamar CA, Bhat GK, Mahesh VB, Brann DW. (1999). Evidence that neuronal nitric oxide synthase but not heme oxygenase increases in the hypothalamus on proestrus afternoon. Neuroendocrinology. 70 360-7.
Laudet V, Gronemeyer H. (2002).The nuclear receptors factsbook . London: Academic Press.

Lindzey J, Crews D. (1986). Hormonal control of courtship and copulatory behavior in male Cnemidophorus inornatus, a direct sexual ancestor of a unisexual, parthenogenetic lizard. Gen Comp Endocrinol 64 411–18.

Lindzey J, Crews D. (1988). Psychobiology of sexual behavior in a whiptail lizard, Cnemidophorus inornatus. Horm Behav 22 279–93.

Lorrain DS, Matuszewich L, Friedman RD, Hull EM. (1997). Extracellular serotonin in the lateral hypothalamic area is increased during the postejaculatory interval and impairs copulation in male rats. J Neurosci. 17 9361-6.

McCann SM, Haens G, Mastronardi C, Walczewska A, Karanth S, Rettori V, Yu WH (2003). The role of nitric oxide (NO) in control of LHRH release that mediates gonadotropin release and sexual behavior. Curr Pharm Des 9 381-90.

McGinnis MY, Kahn DF. (1997). Inhibition of male sexual behavior by intracranial implants of the protein synthesis inhibitor anisomycin into the medial preoptic area of the rat. Horm Behav. 31 15-23.

McIntyre KL, Porter DM, Henderson LP. (2002). Anabolic androgenic steroids induce age-, sex-, and dose-dependent changes in GABA(A) receptor subunit mRNAs in the mouse forebrain. Neuropharmacology. 43 634-45.

Mallick H, Manchanda SK, Kumar VM. (1996). Beta-adrenergic modulation of male sexual behavior elicited from the medial preoptic area in rats. Behav Brain Res. 74 181-7.

Mani SK, Allen JM, Rettori V, McCann SM, O'Malley BW, Clark JH. (1994). Nitric oxide mediates sexual behavior in female rats. Proc Natl Acad Sci U S A. 91 6468-72.

Martinelli GP, Friedrich VL Jr, Holstein GR. (2002). L-citrulline immunostaining identifies nitric oxide production sites within neurons. Neuroscience 114 111–122.

Martini M, Zito G, Viglietti-Panzica C, Panzica G, Balthazart J. (2005). Neuronal Nitric Oxide Synthasein forebrain and mesencephalon is not affected by castration and testosterone therapy in Japanese quail. Program No.320.1.2005 Abstract Viewer. Washington, DC: Society for Neuroscience.

Matuszewich L, Lorrain DS, Trujillo R, Dominguez J, Putnam SK, Hull EM. (1999). Partial antagonism of 8-OH-DPAT'S effects on male rat sexual behavior with a D2, but not a 5-HT1A, antagonist. Brain Res. 820 55-62.

Mendelson SD, McEwen BS. (1990). Testosterone increases the concentration of [3H]8-hydroxy-2-(di-n-propylamino)tetralin binding [corrected] at 5-HT1A receptors in the medial preoptic nucleus of the castrated male rat. Eur J Pharmacol.;181 329-31.

Michael RP, Zumpe D, Bonsall RW. (1990). Estradiol administration and the sexual activity of castrated male rhesus monkeys (Macaca mulatta). Horm Behav. 24 71-88.

Mouton PR. (2002). Principle and Practices of Unbiased Stereology. Baltimore: Johns Hopkins University Press. page 232.

Paredes RG. (2003). Medial preoptic area/anterior hypothalamus and sexual motivation. Scand J Psychol. 44 203-12.

Pehek EA, Warner RK, Bazzett TJ, Bitran D, Band LC, Eaton RC, Hull EM. (1988). Microinjection of cis-flupenthixol, a dopamine antagonist, into the medial preoptic area impairs sexual behavior of male rats. Brain Res. 443 70-6.

Penatti CA, Porter DM, Jones BL, Henderson LP. (2005). Sex-specific effects of chronic anabolic androgenic steroid treatment on GABA(A) receptor expression and function in adolescent mice. Neuroscience. 135 533-43.

Pu S, Xu B, Kalra SP, Kalra PS. (1996). Evidence that gonadal steroids modulate nitric oxide efflux in the medial preoptic area: effects of N-methyl-D-aspartate and correlation with luteinizing hormone secretion. Endocrinology 137 1949–55.

Putnam SK, Sato S, Hull EM. (2003). Effects of testosterone metabolites on copulation and medial preoptic dopamine release in castrated male rats. Horm Behav. 44 419-26.

Putnam SK, Sato S, Riolo JV, Hull EM. (2005). Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats. Horm Behav. 47 513-22.

Rasband WS. (1997–2005). ImageJ [Internet]. US National Institutes of Health, Bethesda. Available from: http:// rsb.info.nih.gov/ij/ In particular, Optical Density Calibration [Modified 2004 Nov 17; cited 2005 Jan 05]. Available from: http://rsb.info.nih.gov/ij/docs/examples/calibration/

Ratnasooriya WD, Dharmasiri MG, Wadsworth RM. (2000). Reduction in libido and fertility of male rats by administration of the nitric oxide (NO) synthase inhibitor N-nitro-l-arginine methyl ester. Int J Androl 23 187–91.

Reilly CM, Zamorano P, Stopper VS, Mills TM. (1997). Androgenic regulation of NO availability in rat penile erection. J Androl 18 110-15.

Retana-Marquez S, Salazar ED, Velazquez-Moctezuma J. (1996). Effect of acute and chronic stress on masculine sexual behavior in the rat. Psychoneuroendocrinology. 21 39-50.

Reynoso R, Mohn C, Retory V, Szwarcfarb B, Carbone S, Rondina D, Moguilevsky JA. (2002). Changes in the effect of testosterone on hypothalamic nitric oxide synthetase during sexual maturation. Its relationship with GnRH release. Neuro Endocrinol Lett 23 101–4.

Rodriguez-Manzo G, Lopez-Rubalcava C, Hen R, Fernandez-Guasti A. (2002). Participation of 5-HT(1B) receptors in the inhibitory actions of serotonin on masculine sexual behaviour of mice: pharmacological analysis in 5-HT(1B) receptor knockout mice. Br J Pharmacol. 136 1127-34.

Sanderson NS, Weissler E, Crews D. (2005). The nitric oxide synthase inhibitor L-NAME suppresses androgen-induced male-like pseudocopulatory behavior in whiptail lizards. Brain Res 1052:236–239.

Sanderson NS, Le BD, Crews D. (2006). Testosterone induction of male-typical sexual behavior is associated with increased preoptic NADPH diaphorase and citrulline production in female whiptail lizards. J Neurobiol 66 1156-63.

Santos AM, Ferraz MR, Teixeira CV, Sampaio FJ, da Fonte Ramos C. (2004). Effects of undernutrition on serum and testicular testosterone levels and sexual function in adult rats. Horm Metab Res. 36 27-33.

Sato Y, Horita H, Kurohata T, Adachi H, Tsukamoto T. (1998). Effect of the nitric oxide level in the medial preoptic area on male copulatory behavior in rats. Am J Physiol. 274(1 Pt 2):R243-7.

Sato S, Braham CS, Putnam SK, Hull EM. (2005). Neuronal nitric oxide synthase and gonadal steroid interaction in the MPOA of male rats: co-localization and testosterone-induced restoration of copulation and nNOS-immunoreactivity. Brain Res. 1043 205-13.

Sato T, Matsumoto T, Kawano H, Watanabe T, Uematsu Y, Sekine K, Fukuda T, Aihara K, Krust A, Yamada T, Nakamichi Y, Yamamoto Y, Nakamura T, Yoshimura K, Yoshizawa T, Metzger D, Chambon P, Kato S. (2004). Brain masculinization requires androgen receptor function. Proc Natl Acad Sci U S A 101 1673-78.

Sato SM, Wersinger SR, Hull EM. (2007). The effects of nitric oxide-cGMP pathway stimulation on dopamine in the medial preoptic area and copulation in DHT-treated castrated male rats. Horm Behav. (in press).

Scaletta LL, Hull EM. (1990). Systemic or intracranial apomorphine increases copulation in long-term castrated male rats. Pharmacol Biochem Behav. 37 471-5.

Scordalakes EM, Shetty SJ, Rissman EF. (2002). Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. J Comp Neurol 453 336-44

Service G, Woodside B. (2007). Inhibition of nitric oxide synthase within the medial preoptic area impairs pup retrieval in lactating rats. Behav Neurosci 121 140-7.

Simerly RB. (1998). Organization and regulation of sexually dimorphic neuroendocrine pathways. Behav Brain Res. 92 195-203.

Simerly RB, Swanson LW. (1987). The distribution of neurotransmitter-specific cells and fibers in the anteroventral periventricular nucleus: implications for the control of gonadotropin secretion in the rat. Brain Res. 400 11-34.

Simerly RB, Swanson LW. (1988). Projections of the medial preoptic nucleus: a Phaseolus vulgaris leucoagglutinin anterograde tract-tracing study in the rat. J Comp Neurol. 270 209-42.

Simerly RB, Swanson LW, Gorski RA. (1984). Demonstration of a sexual dimorphism in the distribution of serotonin-immunoreactive fibers in the medial preoptic nucleus of the rat. J Comp Neurol. 225 151-66.

Simon P. (2003). Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19 1439-40.

Singer CA, Pang PA, Dobie DJ, Dorsa DM. (1996). Estrogen increases GAP-43 (neuromodulin) mRNA in the preoptic area of aged rats. Neurobiol Aging. 17 661-3.

Singh R, Pervin S, Shryne J, Gorski R, Chaudhuri G. (2000). Castration increases and androgens decrease nitric oxide synthase activity in the brain: physiologic implications. Proc Natl Acad Sci U S A 97 3672-77.

Smeets WJ, Alonso JR, Gonzalez A. (1997). Distribution of NADPH-diaphorase and nitric oxide synthase in relation to catecholaminergic neuronal structures in the brain of the lizard Gekko gecko. J Comp Neurol 377 121–41.

Stenberg D (2007) Neuroanatomy and neurochemistry of sleep. Cell Mol Life Sci (in press)

Sumner BE, Fink G. (1998). Testosterone as well as estrogen increases serotonin2A receptor mRNA and binding site densities in the male rat brain. Brain Res Mol Brain Res. 2 205-14.

Thornton JW. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proc Natl Acad Sci U S A. 98 5671-6.

Tin-Tin-Win-Shwe, Mitsushima D, Kimura F. (2002). Profiles of in vivo gamma-aminobutyric acid release in the medial preoptic area of intact and castrated male rats. Neuroendocrinology. 76 290-6.

Tomaselli L, Endoh A, Dodge J, Badura LL. (2001). Regulation of norepinephrine in the medial preoptic area of Siberian hamsters by gonadal steroids. Neuroendocrinology. 74 69-76.

van de Poll NE, van Zanten S, de Jonge FH. (1986). Effects of testosterone, estrogen, and dihydrotestosterone upon aggressive and sexual behavior of female rats. Horm Behav 20 418–31.

Wade J, Crews D. (1991). The effects of intracranial implantation of estrogen on receptivity in sexually and asexually reproducing female whiptail lizards, *Cnemidophorus* inornatus and Cnemidophorus uniparens. Horm Behav 25 342–53.

Wade J, Huang JM, Crews D. (1993). Hormonal control of sex differences in the brain, behavior and accessory sex structures of whiptail lizards (*Cnemidophorus* species). J Neuroendocrinol 5 81–93.

Wood RI (1997). Thinking about networks in the control of male hamster sexual behavior. Hormones and Behavior 32 40-5.

Wood RI. (1998) Integration of chemosensory and hormonal input in the male Syrian hamster brain. Ann N Y Acad Sci 855 362-72.

Woolley SC, Crews D. (2003). Species differences in the regulation of tyrosine hydroxylase in Cnemidophorus whiptail lizards. J Neurobiol. 60 360-8.

Yoo MJ, Searles RV, He JR, Shen WB, Grattan DR, Selmanoff M. (2000). Castration rapidly decreases hypothalamic gamma-aminobutyric acidergic neuronal activity in both male and female rats. Brain Res. 878 1-10.

Young LJ, Lopreato GF, Horan K, Crews D. 1994. Cloning and in situ hybridization analysis of estrogen receptor, progesterone receptor, and androgen receptor expression in the brain of whiptail lizards (*Cnemidophorus uniparens* and *C. inornatus*). J Comp Neurol 347 288–300.

Young LJ, Nag PK, Crews D. (1995). Regulation of estrogen receptor and progesterone receptor messenger ribonucleic acid by estrogen in the brain of the whiptail lizard (Cnemidophorus uniparens). J Neuroendocrinol. 1995 Feb;7(2):119-25.

Zhang L, Ma W, Barker JL, Rubinow DR. (1999). Sex differences in expression of serotonin receptors (subtypes 1A and 2A) in rat brain: a possible role of testosterone. Neuroscience. 94 251-9.

Zumpe D, Bonsall RW, Michael RP. (1993). Effects of the nonsteroidal aromatase inhibitor, fadrozole, on the sexual behavior of male cynomolgus monkeys (*Macaca fascicularis*). Horm Behav. 27 200-15.

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